Study on Dietary Factors Pertinent to the Pathogenesis of Heart Failure in Fast-growing Commercial Broilers

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ABSTRACT

A series of seven experiments were conducted to evaluate the risk of acute (sudden death syndrome; SDS) or chronic (congestive heart failure; CHF) heart failure associated with dietary over-supplementation of vitamin A, vitamin D_3, vitamin E, vitamin C or cardiotoxic factors present in meat meal. The risk of heart failure associated with the above mentioned dietary factors was tested followed by gross, microscopic, ultrastructural and biochemical investigation for mechanisms associated with mentioned risk factors. Simultaneously, the molecular mechanisms underlying the deterioration of heart function in fast-growing commercial broilers were studied. Each compound was tested separately at a concentration higher than the recommended levels. The basic experimental unit comprised groups of 40 to 50 day old male broiler chickens at the start of experiment. Lowered thermal brooding temperature protocol, an approach resulting in clinical manifestation of heart failure in practically all broilers predisposed to heart disease, was used.

Broilers fed the vitamin D_3 enriched diet were 2.5 fold more likely to succumb to acute heart failure ($p<0.05$). Simulated stress challenge with epinephrine revealed that broilers fed excess of vitamin D_3 were more susceptible to ventricular arrhythmia. The risk of CHF was higher ($P<0.05$) in broilers fed the vitamin D_3, vitamin A and methanol soluble extract from meat meal enriched diets as compared to groups fed the control diet. The incidence of CHF in broilers fed the diet fortified with vitamin E was not significantly different as compared to the control group, whereas supplementation of vitamin C in the diet tended ($p=0.10$) to reduce the incidence of CHF. The level of malondialdehyde equivalents, an indicator of lipid peroxidation, was significantly higher ($p<0.05$) in myocardium of broilers developing CHF irrespective of dietary factors. Antioxidant vitamins (E and C) did not prevent lipid peroxidation in broilers developing CHF.

In conclusion, the present findings indicate that over-supplementation of vitamin A and D_3 increases the risk of heart failure in broilers. Meat meal contains some unknown

cardiotoxic factors, capable of precipitating heart conditions in susceptible broilers. Oxidative stress is involved in the pathogenesis of heart failure in broilers, but supplementation of antioxidant vitamins did not prevent oxidative damage in broilers that developed CHF. The oversupplementation of vitamins (A and D_3) should not be encouraged in broilers diet as they may increase the economic losses to broilers industry subsequent to heart related mortalities/morbidities.

Key words: Broiler; Heart Failure; Meat Meal; Oxidative Stress; Vitamin

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BW	Body Weight
CHF	Congestive Heart Failure
CK	Creatine Kinase
Cr	Creatine
CrP	Creatine Phosphate
ECG	Electrocardiography
FM	Fish Meal
HA	Heterocyclic Amines
Hb	Hemoglobin
HPLC	High Performance Liquid Chromatography
HPS	His–Purkinje System
HR	Heart Rate
LDH	Lactate Dehydrogenase
MDA	Malondialdehyde
MM	Meat Meal
NAD	Nicotine Adenine Dinucleotide
NSVT	Non Sustained Ventricular Tachycardia
pCO_2	Partial Pressure of Carbon Dioxide
PDH	Pyruvate Dehydrogenase
pO_2	Partial Pressure of Oxygen
PVC	Premature Ventricular Contractions
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RR	Respiration Rate
SDS	Sudden Death Syndrome
TBARS	Thiobarbituric Acid Reactive Substances

TCA	Tricarboxylic Acid Cycle
α-KGDH	Alpha-Ketoglutarate Dehydrogenase

1. INTRODUCTION

In broiler industry genetic selection is primarily focused on rapid and maximum weight gain with improved feed conversion efficiency. This has resulted in some specific health problems in broilers. In particular, two significant cardiovascular related health effects include acute (sudden death syndrome: SDS) and chronic (congestive heart failure: CHF, associated with hypoxemia, cyanosis and ascites) heart failure. SDS, cyanosis, and ascites in modern fast-growing broiler chickens are among the major causes of economic losses due to morbidity, mortality and/or condemnations. A survey by Maxwell and Robertson (1997) estimated an annual incidence of 4.7% for ascites with global costs around $1 billion per annum. The incidence of SDS has been reported in the range of 0.8% to 9.62% depending on management conditions followed at the poultry farm (Gardiner *et al.,* 1988; Scheideler *et al.,* 1995; Maxwell and Robertson, 1998). In Canada, ascites and cyanosis-related chicken carcass condemnation at the processing plant was 12.82% of total condemnations in 2006 (Agriculture and Agri-Food Canada, 2007).

Cumulatively, heart related conditions account for 60 to 90% of all mortalities and morbidities in commercial broiler flocks. In a single year in Canada heart disease causes death of more than 15 million broilers with estimated losses between $30 and $33 million (based on 2007 market prices) (Personal Communication). Dietary risk factors are presumed to directly trigger 20 to 40% of all losses due to heart failure. Attempts to reduce losses triggered by dietary factors may help to save at least $6 million annually.

Recent research findings on the etiology of heart disease associate several dietary factors (vitamin A, D, C and E over-supplementation and heterocyclic amines) with the pathogenesis of heart failure in humans and laboratory animals (Dubuisson *et al.,* 2001; Colbert, 2002; Liu and Tan, 2002; Villar-Patino *et al.,* 2002; Walentynowicz

et al., 2004; Lorenzoni and Ruiz-Feria, 2006). Similar dietary factors may induce heart failure in fast-growing broilers given the similarities in clinical symptoms, hemodynamic changes, biochemical and pathological aspects of heart failure between mammals and broiler chickens. Hence, this research program undertook an investigation directed towards the role of dietary risk factors in the pathogenesis of heart conditions in fast-growing commercial broilers. We designed seven experiments to elucidate the risk associated with vitamin A, D_3, C and E over supplementation (these vitamins are often over supplemented in broiler feeds) and for potentially cardiotoxic compounds present in meat meal. The analyses involved comprehensive clinical and pathological investigations (gross and microscopic), including an examination of the ultra-structural and biochemical basis of the myocardial defects associated with the dietary manipulations with attempts to understand the molecular mechanisms underlying the development of heart failure in broilers.

2. LITERATURE REVIEW

2.1. Metabolic Disorders as Outcomes of Genetic Selection

During the last few decades, the broiler industry has witnessed tremendous improvement in broiler growth and feed conversion efficiency. Broiler chicken growth rates and feed conversion efficiencies 50 years ago would require double the farm capacity and quadruple the feed requirements to produce the same quantity of meat relative to the modern broiler genotype (Flock *et al.,* 2005). The modern broiler reaches a body weight twice its 1957 counterpart with 20% less feed in half the time (Havenstein *et al.,* 2003).

This genetic selection has resulted in broiler strains that have superior performance, but also has predisposed broilers to higher incidences of specific physiological insufficiencies. The history of metabolic disorders dates back to the last few decades during which poultry breeding programmes focused on fast growth and better feed conversion efficiency. The fatty liver syndrome was one of the major causes of mortalities in the 1960s and 1970s, but not a problem anymore subsequent to improved nutritional strategies (Whitehead, 2000). Today, the major metabolically

related conditions observed in broilers include leg problems or heart related disorders. Predisposition to heart failure is one of the most prominent systemic weaknesses commonly seen in commercial broiler flocks. The focus of breeding programmes on traits of economic importance (i.e. growth rate, feed conversion efficiency and age of slaughter) has ignored the traits critical for broiler welfare.

2.1.1. Heart Failure in Broilers

The main heart related problems in modern broiler flocks are observed either in the form of acute heart failure or chronic heart failure. Today's commercial broiler has compromised heart function and the heart is unable to meet the demand for increased oxygen due to increased body mass and rapid growth (Julian, 2007b). In some broilers, this results in the development of congestive heart failure, initially observed as cyanosis of combs and wattles, and terminally as ascites (abnormal accumulation of fluid in the abdominal cavity).

Fast-growing broilers are inherently predisposed to heart disease with males at greater risk than females (Olkowski and Classen, 1998a). Various cross-sectional studies examining clinical parameters such as blood gas analysis, echocardiography, electrocardiography and necropsy suggest that a large population of broilers are either at a risk of heart failure with various subclinical problems or show signs of heart failure (Odom *et al.,* 1992; Owen *et al.,* 1995; Olkowski *et al.,* 1999; 2003b; 2007a). Studies in our laboratory directly link impaired heart function to fast-growing broilers since birds not selected for fast growth, i.e. leghorn chickens, show no signs of subclinical or clinical heart failure (Olkowski *et al.,* 2005b). Furthermore, slowing of growth by dietary feed restriction decreases the risk of acute and chronic heart failure in broilers (Acar *et al.,* 1995; Olkowski *et al.,* 2007a).

2.1.1.1. Acute Heart Failure

Acute heart failure in fast-growing broilers manifests as sudden death syndrome (SDS) or 'flip-over syndrome' where young, healthy, fast-growing broilers die suddenly with no discernible cause evident upon post mortem examination (Olkowski and Classen, 1995). This disorder is not restricted to broilers with reports of SDS in a large number of species including human, horses, calves and turkeys. In human infants SDS is known as sudden infant death syndrome. Hemsley (1965) was one of the first authors to describe "edema of the lung" as a separate cause of mortality in broilers with unknown etiology. The sudden death syndrome has been described by various names such as flip-over / acute death syndrome / fatal syncope / lung edema / heart attack (Brigden and Riddell, 1975; Ononiwu *et al.*, 1979; Olkowski and Classen, 1995).

SDS incidence is associated with rapid growth rate and mortality associated with this condition is reduced drastically by growing broilers at a slower rate (Bowes *et al.*, 1988). In mixed sex flocks, male represents more than 70% of the total mortalities due to SDS (Olkowski and Classen, 1998a).

2.1.1.1.1. Pathophysiology and Electrophysiology

The underlying mechanism of death in broilers succumbing to SDS is fatal cardiac arrhythmia and birds die of ventricular fibrillation (Olkowski and Classen, 1997; Nain *et al.*, 2007). Left ventricular hypertrophy has been associated with increased risk of ventricular arrhythmias and sudden cardiac death in humans (Saadeh and Jones, 2001). Cardiac hypertrophy in broilers is a compensatory response to a variety of physiological or pathological stimuli to myocardium. However, prolonged hypertrophic responses may lead to arrhythmia, heart failure and sudden death.

A variety of factors including nutrition, genetic, and environmental have been linked to the incidence of SDS (Scheideler *et al.*, 1995; Gonzales *et al.*, 1999; Imaeda, 1999; 2000). However, the triggering event that leads to the catastrophic arrhythmia and sudden death in broilers remains unknown. Recent observations from our laboratory

suggest that the presence of stressful stimuli along with pathological changes in the myocardium and His-Purkinje system may contribute significantly to the generation of catastrophic arrhythmia, leading to sudden death (Olkowski *et al.*, 2007b; Nain *et al.*, 2007).

2.1.1.2. Chronic Heart Failure

Chronic/congestive heart failure (CHF) in modern broilers is observed in the form of hypoxemia, cyanosis and ascites in fast-growing broilers. With the high metabolic demand associated with rapid growth, heart function in many broilers is marginally capable of providing enough blood flow to sustain the basic oxygen requirements. Consequently, many fast-growing broilers show cyanotic combs, wattles and skin, which are clear signs of hypoxemia. Additionally, the fast-growing broilers have a lower oxygen requirement per kg of body weight, a result of lowered thyroxin level (Malan *et al.*, 2003), but they become hypoxemic due to their greater body mass and impaired heart function (Nain *et al.*, 2008b). Electrocardiographic studies by Olkowski *et al.* (1997) revealed that 30% of the broilers may be at a risk of developing heart failure. A relatively large proportion of commercial broilers show evidence of sub-clinical heart disease (Olkowski *et al.*, 1998; 1999; 2005b). Echocardiographic examination of broilers with CHF revealed lowered left ventricular fractional shortening, indicating impaired contractile functions in the ventricular myocardium of such birds (Olkowski *et al.*, 2005a; Deng *et al.*, 2006).

The literature sometimes considers ascites a disease. However, ascites is only a sign of congestive heart failure in broilers. Interestingly, ascites was initially observed in broilers raised at high altitude above 1500 m, but it is no longer restricted to high altitude (Wiseman and Garnsworthy, 1999). A number of conditions lead to ascites in animals (cirrhosis, heart failure, veno-occlusive disease and pericarditis) but in broilers it is subsequent to venous congestion as a result of impaired heart function.

2.1.1.2.1. Cardiovascular Pathophysiology

Normal heart function is essential to sustain oxygen requirement of the body. Fast growth in broilers results in increased muscle mass, which puts an extra burden on the heart to increase cardiac output. Those broilers unable to meet this increased demand develop a state of hypoxemia (Fedde *et al.,* 1998; Olkowski *et al.,* 2005b). This hypoxemia is associated with circulatory insufficiency as a result of progressive bradycardia observed in fast-growing broilers (Olkowski and Classen, 1998b; Olkowski *et al.,* 2007a). This deterioration of heart function in these broilers showing early signs of heart failure results in congestion of the venous system, leading to exudation of plasma from blood to body cavities and development of ascites. Additionally, studies have shown that cardiopulmonary parameters are extremely unfavorable in modern fast-growing broilers leading to higher susceptibility to development of CHF and ascites (Hassanzadeh *et al.,* 2005).

In comparison to breeds of chickens not selected for rapid growth, such as leghorns, fast-growing broilers have relatively smaller structural and functional hearts, and reduced capacity of the left ventricle (Martinez-Lemus *et al.,* 1998; Olkowski *et al.,* 2005a). Some studies consider the role of right ventricular failure in the development of ascites (Julian *et al.,* 1987; McGovern *et al.,* 1999). However, others suggest that right ventricular failure is subsequent to pulmonary hypertension, due to anatomically or functionally inadequate pulmonary vascular capacity in fast-growing commercial broilers (Wideman *et al.,* 2007). In ascitic broilers, right and left ventricular hypertrophy is observed and right ventricular hypertrophy may be secondary to left ventricular dysfunction and pulmonary hypertension as suggested by Olkowski (2007). The dilation of the left ventricle can not be a result of pulmonary hypertension or right ventricular failure but it is most probable that all these changes i.e. pulmonary hypertension, right ventricular failure are subsequent to left ventricular failure. Hence, it can be interpreted that this right ventricular failure hypothesis is devised by simply looking at the dilation of ventricular chambers, which is more apparent on the right side because of its anatomical structure and already existing thin wall. In addition, other supporting arguments provided

in the support of this hypothesis is the pulmonary hypertension observed in the ascitic broilers. However, some authors suggest that this pulmonary hypertension is a result of left atrio-valve regurgitation, leading to stasis of blood back in the left atrium and pulmonary circulation, a result of degeneration of left atrio-ventricular valve (Olkowski *et al.,* 2005a).

2.1.1.2.2. Heart Pathology

Heart failure in broilers results from a variety of morphological, molecular and biochemical changes in the cardiomyocytes and extra-cellular matrix components of the myocardium. Studies using *ad libitum* feeding and cold stress models revealed ventricular hypertrophy and dilation, the most common findings in all birds showing congestive heart failure (Wu *et al.,* 2003; Julian, 2007b; Olkowski, 2007).

Post mortem examination on birds with congestive heart failure reveals abnormal accumulation of ascitic fluids in abdominal cavity along with gross dilation of the ventricular chambers, pathological changes of the atrioventricular valve, pericardial effusion and adhesions (Olkowski *et al.,* 1998; Nain *et al.,* 2008b). The gross lesion observed on left atrio-ventricular valves consists of nodules associated with annulus, valve cups, and chordae tendinae (Olkowski *et al.,* 1998). In another study by Olkowski *et al.* (2003b) excessive pericardial effusions, local adhesion between parietal and visceral pericardium and fibrous deposits on visceral pericardium were also observed.

Ultra structural examination of the affected myocardium revealed mitochondrial vacuolization and disintegration, loss of myofibrillar components, and changes in extra cellular matrix components (Olkowski *et al.,* 2001). Recently, Li *et al.* (2006) demonstrated calcium deposits in the ventricular myocardium of ascitic broilers.

2.1.1.2.3. Role of Oxidative Stress in Chronic Heart Failure

The heart requires a constant supply of oxygen for normal cardiac functions. However, the role of oxygen and oxygen associated processes are complex in the myocardium where they are generally beneficial but sometimes can contribute to cardiac dysfunction and ultimately heart failure (Andreka *et al.,* 2004; Giordano, 2005).

An imbalance between reactive oxygen/nitrogen species (ROS/RNS) production and antioxidant defenses leads to oxidative stress (Orrenius *et al.,* 2007). Growing evidence suggests that ROS/RNS plays a critical role in heart failure etiology. In all organisms, oxidation is a normal part of metabolism, but during this process various ROS/RNS are produced. The generated ROS/RNS have some metabolic significance in normal physiological functions. However, as ROS/RNS production exceeds antioxidant defenses, these compounds are able to alter properties of cellular macromolecules such as nucleic acids, phospholipids and proteins, which can lead to myocardial dysfunctions.

Elevated ROS levels have been demonstrated in the failing human heart (Sam *et al.,* 2005). Maxwell and Robertson (1996) demonstrated increased hydrogen peroxide activity in the cardiomyocytes from failing hearts in broilers. Membrane lipid peroxidation can alter properties crucial for maintenance of normal functions of the cell and sub cellular components (mitochondria and sarco-endoplasmic reticulum). In broilers with congestive heart failure, evidence of calcium overload in these sub-cellular components exists (Maxwell *et al.,* 1993; Li *et al.,* 2006). In addition there is some evidence of breakdown and release of the contractile proteins of the myocardium e.g. myosin and troponin T into circulation (Maxwell *et al.,* 1994; 1995). The role of oxidative stress has long been debated in the pathogenesis of heart failure in humans and animals models of cardiomyopathy, but not much work has been done to determine the possible involvement of this oxidative stress in heart failure etiology in broilers and thus warrants detailed investigation.

2.1.1.2.4. Energy Metabolism in Chronic Heart Failure

The heart functions to convert chemical energy (high energy phosphates) into mechanical work in the form of cardiac output. The heart is one of the most energy demanding organs of the body. Mitochondria play a central role in providing a constant ATP supply to the myocardium, occupying 30% of cardiomyocyte volume. The loss of contractile function of the heart is associated with mitochondrial inability to supply ATP to the myocardium, leading to a state of energy deprivation in the heart (for review see Stanley *et al.,* 2005). Deterioration of heart pump function has been linked to the insufficiency of high energy phosphates in human and animal models of cardiomyopathy (Liao *et al.,* 1996; Sharov *et al.,* 1998; 2000; Hansch *et al.,* 2005; Nakae *et al.,* 2005). Not much is known about the etiology and mechanism for insufficiency of high energy phosphate levels in dilated cardiomyopathy. Some authors suggest depressed activity of electron transport chain components (Buchwald *et al.,* 1990; Casademont and Miro, 2002). Patients with dilated cardiomyopathy exhibit altered myocardial metabolism characterized by reduced uptake and metabolism of fatty acids (Taylor *et al.,* 2001; Davila-Roman *et al.,* 2002). Studies using animal models of heart failure revealed decreased gene expression encoding β-oxidation of fatty acids during heart failure causing a shift similar to that of fetal heart in cardiac mitochondria, with glucose being a major substrate instead of fatty acids (Buttrick *et al.,* 1994; Barger and Kelly, 1999).

The one of the earliest identified biomarker of already well established pathophysiological change in declining heart pump function in fast-growing broilers is a declining heart rate (Olkowski and Classen, 1998b; Druyan *et al.,* 2007). This bradycardia is concomitantly accompanied by reduced ventricular wall motion and reduced ventricular fractional shortening and hence lowered cardiac output (Olkowski *et al.,* 1999; Olkowski *et al.,* 2005a; Deng *et al.,* 2006). Recently, insufficiency of energy substrate has been linked with this deterioration of heart function in broilers (Olkowski *et al.,* 2007a; Nain *et al.,* 2008b). The mechanisms underlying this

insufficiency of energy substrates have not been identified and warrant detailed investigation.

2.1.1.3. Factors Predisposing to Heart Failure

The fast-growing commercial broilers are inherently predisposed to heart conditions. The main factor that predisposes broilers is altered physiology as a result of fast growth. Secondary causes include a variety of environmental factors to which a broiler is exposed.

2.1.1.4. Broiler Physiology

Subsequent to the intensive focus of broiler breeder companies for traits of economic importance, commercial broilers generally have certain cardiopulmonary disturbances that lead to increased risk of heart failure. Modern broilers rapidly accrue body mass without a relative increase in cardiac performance (Julian, 1993). This results in a state of imbalance between oxygen supply and demand. Fast-growing broilers have lower blood pO_2 and hemoglobin oxygen saturation % as compared to broilers considered to be resistant to heart failure i.e. feed restricted broilers and leghorn chickens (Olkowski *et al.*, 2005b; Nain *et al.*, 2008b). These observations suggest that preexisting pathophysiological disturbances predispose the modern fast-growing broilers to the risk of heart failure. Interestingly, studies demonstrated lower plasma thyroid hormone level and low arterial pO_2 and high pCO_2 pressure in modern fast-growing broilers as compared to the slow growing lines (Scheele *et al.,* 1992; Malan *et al.,* 2003). Additionally, studies have linked the development of congestive heart failure and ascites with altered thyroid function (Scheele *et al.,* 1992; Decuypere *et al.,* 1994; Malan *et al.,* 2003).

Cardiac index, a parameter relating cardiac output to the body weight, is lower in fast-growing broilers and in broilers with congestive heart failure as compared to leghorn chickens or feed-restricted broilers (Olkowski *et al,.* 2005b). Feed restricted broilers have normal hearts and heart function, which alleviates the problem of acute or

chronic heart failure, but this comes at the expense of a decrease in growth rate (Acar *et al.,* 1995).

2.1.1.5. Environmental Factors

Environment is a major contributor to increased broiler susceptibility to CHF and ascites. In particular, temperature and lighting programmes show strong association with increased risk of heart failure.

2.1.1.5.1. Brooding Temperature

Lowering the environmental temperature is routinely practiced to study heart failure in broilers (Buys *et al.,* 1999; Lorenzoni and Ruiz-Feria, 2006; Olkowski *et al.,* 2007a). This lowered brooding temperature works by increasing metabolic rate, which results in increased burden on the cardiovascular system. This factor is very effective in increasing the risk of heart failure in broilers.

2.1.1.5.2. Lighting Programmes

Broilers on long day period are at increased risk of heart related problems (Hassanzadeh *et al.,* 2003; Leeson and Summers, 2005), while intermittent lighting programmes decrease the incidence of CHF (Buys *et al.,* 1998). In addition to duration, light intensity can also influence the incidence of metabolic disorders since it affects the bird's activity and feed intake (Leeson and Summers, 2005). Broilers are reluctant to eat during the dark period, resulting in decreased feed intake, growth rate and hence reduced metabolic rate (Hassanzadeh *et al.,* 2003).

2.1.1.6. Nutritional Status

The main aim of poultry nutrition is to optimize production efficiency at minimum input cost. Manipulation of feed supply or composition in broilers can affect the incidence and severity of metabolic disorders.

Qualitative or quantitative feed restriction decreases the incidence of these disorders by slowing the growth rate of broilers. Feed restriction can reduce the risk of acute and chronic heart failure, but the level of feed restriction determines the risk of developing heart failure (Shlosberg *et al.,* 1991; Olkowski *et al.,* 2007a).

Dietary form and composition (pellet feeding, high nutrient dense diet) may also affect the incidence of these metabolic disorders. Mesh feeding decreases the incidence of SDS and CHF in broilers (Leeson and Summers, 2005). Feeding dietary calcium above NRC recommendations increases susceptibility to SDS in broilers (Scheideler *et al.,* 1995). Mollison *et al.* (1984) observed that feeding a high protein (24%) diet decreases the incidence of chronic heart failure. Excess of dietary sodium is linked to increased incidence of ascites in broilers (Shlosberg *et al.,* 1998; Xiang *et al.,* 2004).

2.1.2. Dietary Risk Factors Precipitating Heart Failure

From the above discussion, it is clear that fast-growing broilers are at increased risk of developing heart failure. Hence, presence of any cardiotoxic compound can further increase the risk of heart failure by directly affecting cardiomyocytes or indirectly through extracellular matrix remodeling in the heart.

2.1.2.1. Vitamin Over-supplementation

Excessive intake of vitamin A, D, E, C and niacin were found to be associated with a variety of metabolic lesions and adversely affect heart performance leading to increased risk of heart failure in various mammalian and avian species (Wrzolek, 1985; Colbert, 2002; Liu and Tan, 2002; Walentynowicz *et al.,* 2004; Millemann *et al.,* 2007).

Retinoic acid, a vitamin A metabolite, helps in regulating cardiac form and function during embryogenesis. Over-expression of retinoic acid receptor leads to dilated cardiomyopathy and congestive heart failure in mice (Colbert *et al.,* 1997). Vitamin A and its metabolites have been linked to defective malformation of heart in rats (Mulder *et al.,* 2000). Recently, cardiovascular defects in cattle have been

associated with high levels of vitamin A exposure during embryonic life (Millemann *et al.,* 2007). Also noteworthy is that hypervitaminosis A has been shown to cause ascites in human beings (Croquet *et al.,* 2000; Miksad *et al.,* 2002).

Notably, diet fortification with vitamin A is a common practice in broiler breeders to improve fertility and egg production. Hence, the incidence of heart failure observed in some broiler flocks may be a result of over-supplementation with vitamin A in broiler or broiler breeder diets.

Oversupplementation with vitamin D is found to be associated with heart failure with various microscopic lesions in myocardium in human or animal model of cardiomyopathy (Walentynowicz and Wrzolkowa, 1995; Walentynowicz *et al.,* 2004). Microscopic lesions include disruption of extra-cellular matrix components and fragmentation of myofibrils in cardiomyocytes (Walentynowicz *et al.,* 2004). Hypervitaminosis D produces massive calcium accumulation in mitochondria, which may result in the loss of mitochondrial function in cardiomyocytes in rats (Takeo *et al.,* 1991). Vitamin D toxicity leads to *in situ* loss of myofibrillar protein components in cardiomyocytes by increased proteolytic activity (Walentynowicz and Wrzolkowa, 1995). In cases of hypervitaminosis D, this myofibrillar loss may be preceded by calcium deposition in cardiomyocytes (Wrzolkowa *et al.,* 1991). Hence, damage to cardiomyocytes is most probably by calcium activated peptidases (Walentynowicz and Wrzolkowa, 1995).

Conflicting reports exist with the use of vitamin E and C supplementation and their effect on the incidence of CHF in broilers. Some suggest that vitamin C decreases the CHF incidence in broilers (Al-Taweil and Kassab, 1990), while others suggest neither vitamin E nor C supplementation in broilers have beneficial effects in the prevention of CHF (Bottje *et al.,* 1997; Villar-Patino *et al.,* 2002). Walton *et al.* (2001) reported that supplementing with a combination of vitamin E and C increased the incidence of CHF in broilers. Vitamin C, by its interaction with Fe(II) and Cu(I) may generate reactive oxygen species, leading to cardiac injuries and heart failure (Fisher *et*

al., 2004). Over-supplementation of vitamin E showed detrimental effects on pulmonary artery relaxation and may be a risk factor for development of heart failure (Lorenzoni and Ruiz-Feria, 2006). The human literature also presents conflicting reports about the possible use of vitamin E in treatment of heart failure. Earlier studies suggested beneficial effects with the use of vitamin E in cardiovascular diseases (Singal and Kirshenbaum, 1990; Bauersachs *et al.,* 2001); later some reports found no beneficial effects (Keith *et al.,* 2001; Mak and Newton, 2001; Vivekananthan *et al.,* 2003) and few recent reports suggest vitamin E may increase the risk of heart failure (Lonn *et al.,* 2005; Miller *et al.,* 2005). Additionally, supplementation of vitamin E aggravated the heart damage caused by doxorubicin (Liu and Tan, 2002).

Niacin may be associated with heart failure. An increased blood homocysteine level was associated with niacin supplementation **(**Garg *et al.,* 1999; Basu and Mann, 1997) and a high homocysteine level is associated with cardiovascular disease (Toole *et al.,* 2004; Vollset and Ueland, 2005). Recent *in vitro* studies demonstrated that homocysteine has dose dependent effects on cardiomyocyte viability (Sipkens *et al.,* 2007).

The possibility of vitamin over-supplementation in broiler rations as well as in broiler breeder rations always exists due to a variety of reasons. Firstly, some animal tissues and organs accumulate significant vitamin levels and these tissues may end up in the poultry feed formulations after rendering at the processing plant. Secondly, some vitamins such as A, D, and E are added in diets with the assumption that these are deficient in feed (Julian, 2007a) without prior analysis. This may lead to gross over-formulation of diets as basal levels of these vitamins are always present in broiler diets depending on recommendations and the dietary sources used in feed formulation. Consequently, a high probability exists that some vitamins are present at excessive levels in broiler diets.

Excess dietary levels of these vitamins in broiler diets may increase the risk of cardiac arrhythmia, acute heart failure (SDS) and/or chronic heart failure. Although

these vitamins are supplemented in feeds to promote maximum productivity, excessive levels may actually result in losses to the broiler industry due to enhanced incidence of heart failure and ensuing mortalities. In some cases, a narrow margin between physiological requirements and toxic dose has been observed such that slight over supplementation may initiate adverse effects in susceptible individuals (De Vries, 1997) and lead to heart failure.

2.1.2.2. Factors Associated with Feed Processing

In a number of reports, the etiology of heart failure in laboratory animals and humans has been associated with thermal food processing (Davis *et al.,* 1994; Gaubatz, 1997; Dubuisson *et al.,* 2001). Cooked meat contains a number of mutagenic heterocyclic amines (HAs) including 2-amino-3-methylimidazo quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP), which experimentally produces foci of chronic inflammation with myocyte necrosis, myofibrillar dissolution and disintegration, and dilation of T-tubules in rats **(**Davis *et al.,* 1994).

HAs induced changes in myocardium include mitochondrial swelling and vacuolization along with distortion of their banding pattern (Takahashi *et al.,* 1996). Recent research has shown that some heterocyclic amines found in cooked food preferentially produce DNA damage in cardiomyocytes (Overvik *et al.,* 1991). This issue has been studied in detail in the pathogenesis of cardiomyopathy in humans, because conventional consumption of heterocyclic amines through meat products may lead to damage in human hearts (Gaubatz, 1997). Since cardiomyocytes are terminally differentiated cells that have lost their ability to divide, the capacity to repair DNA damage is a critical factor in proper functioning of damaged cardiomyocytes. These compounds may cause persistent damage to cardiomyocytes, resulting in pathological lesions and compromised heart function (Thorgeirsson *et al.,* 1994; Takahashi *et al.,* 1996).

PhIP, the most potent heterocyclic amine found in cooked meat is bioactivated by o-acetyltransferase, an enzyme with maximum activity in neonatal cardiomyocytes

that decreases with age. This indicates that the young are at greater risk of damage from dietary heterocyclic amines (Dubuisson *et al.*, 2001). From this, we can infer that poultry birds in their growing stage are at greater risk of cardiac damage by heterocyclic amines.

Heterocyclic amines are formed during high-temperature treatment of proteins such as rendering of meat products, which are later supplemented in broilers ration. Thus far, twenty HAs generated in cooked meat have been identified (Bordas *et al.*, 2004). The amounts and types of HAs formed during cooking are attributed to parameters such as time and temperature (Gross *et al.*, 1993; Knize *et al.*, 1998). These HAs are mainly divided into two main classes: 1) aminoimidazoazaarenes and 2) aminocarbolines. The aminoimidazoazaarenes, also known as imidazo quinolines (IQ)-type compounds, are generated from precursors such as glucose, creatine/creatinine and free amino acids at ordinary cooking temperatures (Bordas *et al.*, 2004). In contrast, aminocarbolines, also called pyrolitic HAs, are formed through pyrolitic reactions at temperatures above 300°C. In poultry ration formation of aminoimidazoazaarene HAs are more likely because rendering of animal tissues is carried out by two types of processes: dry rendering with high temperature (135°C) and wet rendering with low temperature (70°-100°C), a range of temperature where chances of formation of aminoimidazoazaarene HAs are greater. Heterocyclic amines detected in various meat samples vary with a range of 0.2-7.7 ng per gram of meat (Warzecha *et al.*, 2004).

In addition to animal protein, vegetable sources (i.e. bean cakes) may contribute to HAs in poultry ration (Lan *et al.*, 2004). Sometimes, soybean is also included in poultry diet up to 15% after heat treatment to remove its antinutrients. Since, all animal by-products used as dietary supplements in poultry are processed at high temperature during rendering process, heterocyclic amines are produced and most likely present in the broilers diet, which may increase the risk of heart failure.

2.1.2.3. Environmental Pollutants

The halogenated cyclic hydrocarbons such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs) and dioxins are structurally related heterocyclic hydrocarbons. These are chemically stable, lipid soluble chemicals that concentrate in fatty tissues and thereby present a hazard in the food chain of animals (Chovancova *et al.,* 2005). Broilers may be exposed as a result of feed contamination subsequent to industrial or agricultural usage.

2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent halogenated aromatic hydrocarbon known, has been associated with cardiovascular toxicity in chickens **(**Ivnitski *et al.,* 2001). Furthermore, these compounds increase the incidence of arrhythmias in developing chickens **(**Fan *et al.,* 2000; Sommer *et al.,* 2005) and heart disease in human beings (Steenland *et al.,* 1999). This arrhythmia may be as a result of increased intracellular Ca^{2+} concentrations and decreased β-adrenergic receptor responsiveness in cardiomyocytes (Canga *et al.,* 1988). TCDD toxicity in marmosets (*Callithrix jacchus*) leads to fibrosis, which may be due to TCDD-mediated increase in transforming growth factor β1 (TGFβ1) (Riecke *et al.,* 2002). However, Yndestad *et al.* (2004) suggested rather than the whole super-family of TGFβ1, activin A might be involved in fibrosis in heart during cardiovascular remodeling.

Aryl hydrocarbon receptor agonists (dioxin) may exert toxic responses similar to those of TCDD. Endothelial cells of the cardiovascular system act as a specific target for bioactivation and toxicity of aryl hydrocarbon receptor agonist in birds (Annas *et al.,* 1998). In one study using Plymouth Rock-Barred and White Leghorn-Babcock chick embryos, dioxin toxicity resulted in a phenotype of dilated cardiomyopathy with symptoms associated with the development of congestive heart failure (Walker and Catron, 2000; Heid *et al.,* 2001). Walker and Catron (2000) also observed that Plymouth Rock-Barred (heavier breed) chick embryos were four to five times more sensitive to this cardiotoxicity than White Leghorn-Babcock. Hence, heavier breeds of chickens are more predisposed to these environmental toxic factors. Therefore, it is

reasonable to speculate that broilers susceptible to heart disease fed diets containing these compounds would be at high risk of fulminant heart failure.

Various contaminants including polychlorinated biphenyls, organochlorine pesticides and polybrominated diphenyl ethers are detected in a number of commercially available fish and vegetable oil dietary supplements (Bocio *et al.,* 2003; Jacobs *et al.,* 2004). PCBs are also detected from grains stored in silos coated with paints (Willett *et al.,* 1985). Residues of organochlorine and PCBs have been detected from oat, barley, wheat, rye and meat samples (Kveseth and Bjerk, 1975).

A significant proportion of broiler diets include animal rendered products. Tallow, cooking fats and oils are frequently the principal sources of dietary fat used in poultry nutrition. Environmental pollutants such as PCB, dioxins, hydrocarbons etc. readily accumulate in adipose tissue of animals (Fromberg *et al.,* 1999; Covaci *et al.,* 2002; Hobbs *et al.,* 2002) as a result of biomagnification. Interestingly, tallow supplementation in broilers diet is associated with defective cardiac sarcoplasmic reticular membrane function and acute heart failure (Chung *et al.,* 1993). Therefore, animal by-product supplements used in poultry diets containing these environmental pollutants may increase the risk of heart failure in broilers.

3. MATERIALS AND METHODS

3.1. Common Research Approach

The basic strategy followed for the thesis research includes *in vivo* trials centered on clinical responses to dietary factors (vitamin A, D, E, and C and methanol soluble factors in meat meal) under controlled experimental conditions. The *in vivo* studies were conducted by using commercial male broiler (Ross X Ross 308) chickens. We performed seven trials, in which each compound was tested separately at a concentration higher (approximately sixteen times for vitamin A, D_3 and E and 2.5 times for putative compounds present in meat meal) than recommended levels.

3.1.1. Animals, Treatments and Management

Male broiler (Ross X Ross 308) chicks were obtained from a commercial flock. The basic experimental unit was comprised of a group of 40 to 50 day-old male broiler chickens. Each experimental unit was replicated twice or thrice to test the risk of acute or chronic heart failure with each dietary ingredient and each experiment was further replicated twice (vitamin A and D, and putative compounds present in meat meal) or thrice (Vitamin E and Cl) over a period of time. All birds were fed *ad libitum* with commercial broiler diet (Table 3.1) and had unrestricted access to water. The birds were housed from day old in an environmentally (temperature and ventilation) controlled rooms (either raised perforated floor pens or pens with litter soil) under constant light. During the first seven days the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 30% (weeks 2, 3) and 40% (weeks 4, 5) lower than that set for normo-thermal brooding temperature (Figure 3.1). This lowered environmental temperature forces birds to increase their metabolic rate, which results in increased burden on the cardiovascular system. This approach is very effective in precipitating heart failure in broilers predisposed to heart conditions.

Table 3.1. Chemical composition of commercial broiler diet.

Chemical Composition	**Guaranteed Analysis**	**% basis**
Crude Protein	Minimum	20.0%
Crude Fat	Minimum	2.0%
Crude Fibre	Maximum	5.0%
Calcium	Actual	0.9%
Phosphorus	Actual	0.7%
Sodium	Actual	0.2%
Selenium	Actual	0.30 mg/kg
Vitamin A	Actual	1500 IU/kg
Vitamin D_3	Actual	5000 IU/kg
Vitamin E	Actual	60 IU/kg

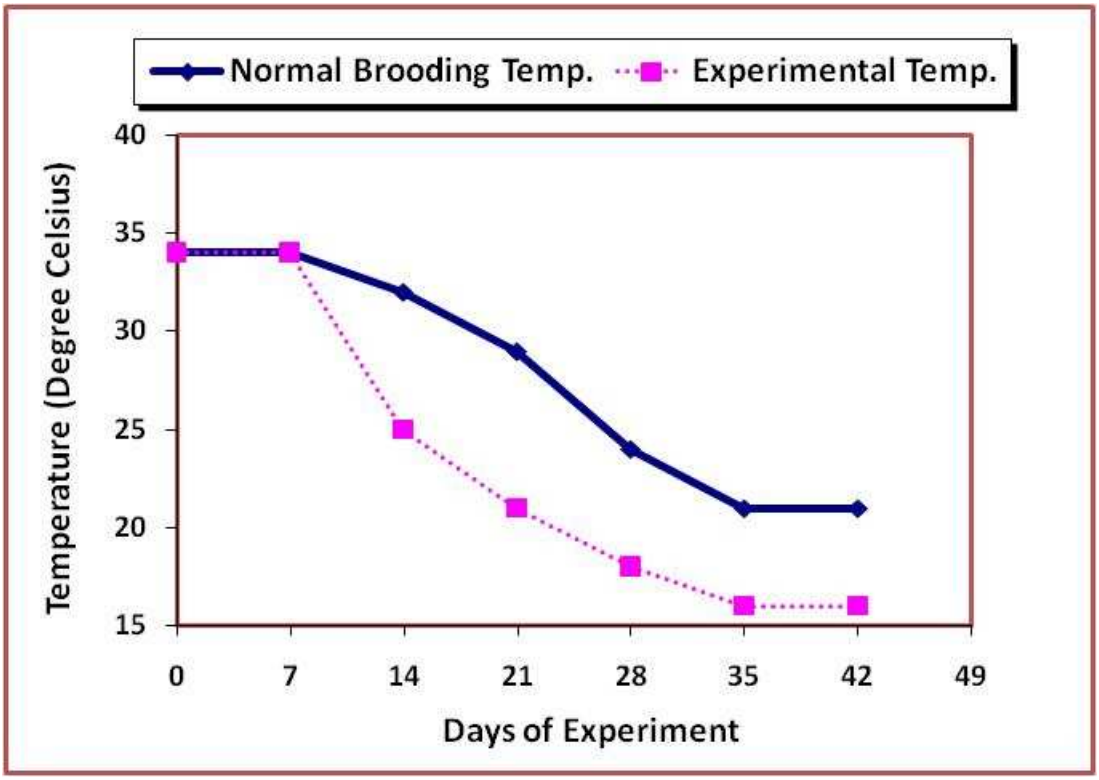

Figure 3.1. Lowered experimental brooding temperature protocol.

Leghorn chickens and feed-restricted slow-growing broilers are considered as resistant to heart failure (Olkowski *et al.*, 2005b; 2007). To explain physiological and biochemical disturbances leading to congestive heart failure in the fast-growing commercial broilers, additional data were collected from 48 leghorns and 39 feed-restricted broilers during fifth (heart rate, respiration rate and blood gas parameters) and sixth (high energy phosphates and enzyme activity measurements in myoocardium) week of age. The birds in feed-restricted group were provided with a diet equal to 70% of the *ad libitum* fed group from age day 7 onwards until the end of experiment (six weeks of age). This feeding regime results in slower growth in the feed restricted group, with body weights lower by 25 to 30% than that of the *ad libitum* fed group (Olkowski *et al.*, 1999; 2005b; 2007a). Our laboratory has used this model routinely to practically eliminate the risk of heart failure to zero and the outcomes in terms of growth rate and body weight are quite consistent.

Experimental protocols were approved by the University of Saskatchewan Animal Care Committee and procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

3.1.2. Clinical Evaluation

A standard experimental procedure was followed, where birds were monitored daily for overt clinical signs of heart disease such as fatigue, exercise intolerance, tachypnea, cyanosis and ascites. Birds were visually assessed for presence of CHF above mentioned clinical signs; if birds showed signs of CHF then these were euthanized and subjected to post-mortem examination.

Bluish discoloration of combs and wattles is known as cyanosis, a condition found to be associated with impaired cardiac function in broilers. In order to access the proportions of birds with cardiac dysfunctions, we counted the number of cyanotic birds in each treatment group. Before doing this measurement, we validated our visual measurements with blood gas parameters to ensure that birds being considered as cyanotic had impaired heart function. For validation purposes, ten birds considered as cyanotic and ten apparently normal were sampled for blood gas analysis. The blood gas analysis revealed that those birds with cyanosis had higher blood pCO_2 and lower pO_2 and hemoglobin O_2 saturation as compared to apparently normal birds (see Figure 3.2).

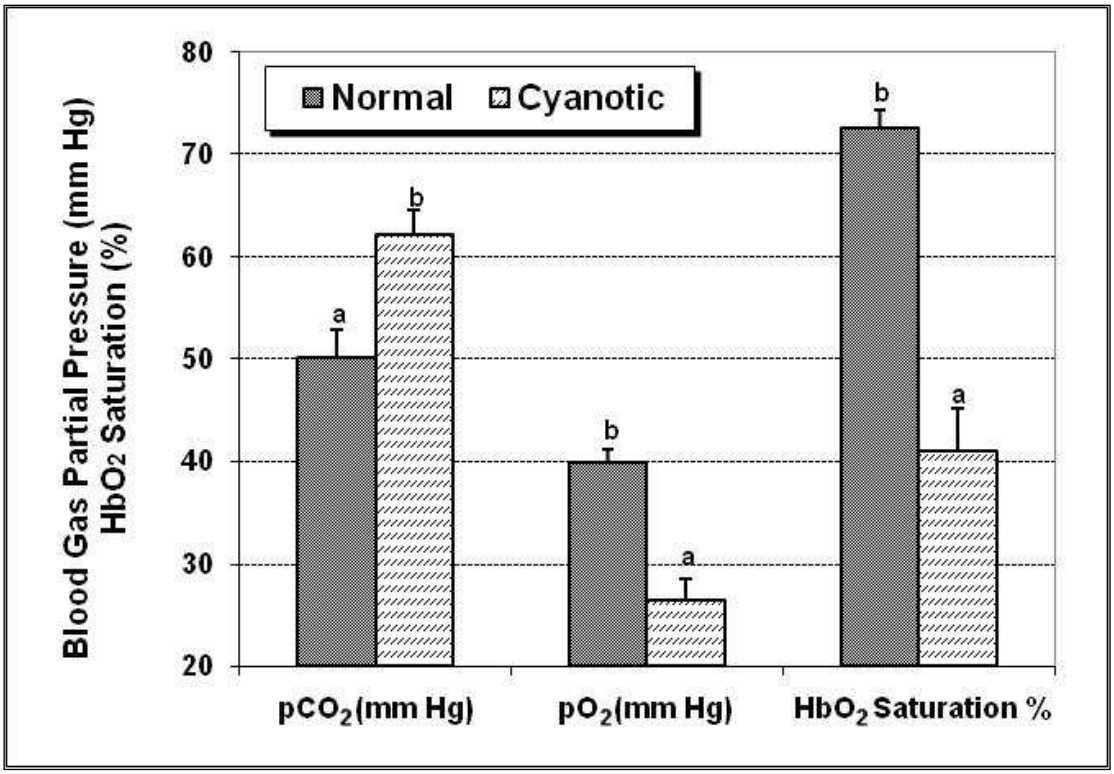

Figure 3.2. Comparison of blood gas parameters in venous blood from broilers showing signs of cyanosis (bluish discoloration of combs and wattles) and from broilers with apparently normal combs and wattles.
[a,b] Adjacent bars with different superscripts are significantly different ($P \leq 0.05$). Significant differences in pCO_2, pO_2 and HbO_2 saturation % between apparently normal birds and birds with cyanotic combs and wattles were assessed with analysis of variance, means were separated using Fisher LSD using microcomputer package NCSS (Hintze, 1995). Values are means ± SE (n=10 birds).

3.1.3. Electrocardiographic Measurements

ECG measurements were obtained during the course of study from randomly selected birds from each group. These measurements were obtained by subcutaneous implantation of ECG probes using bipolar lead II arrangement after induction of light anesthesia using isoflurane at a concentration of 2.0%, delivered by an agent-specific precision vaporizer in birds inhaling pure oxygen (100%) at a flow rate of 1.0 L/min. Following induction, anesthesia was maintained with 1.0% isoflurane. Lead II of the electrocardiogram (ECG) was continuously monitored using a base-apex configuration.

The ECG signals were digitized using a digital data recording unit and software (Mac Lab and Scope 3.3: AD Instruments Pty Ltd, Castle Hill, Australia) and processed using a Macintosh computer. An average of three readings was taken to measure the average heart rate. The ECG data were evaluated for heart rate and normal and abnormal heart electrophysiological patterns. The common cardiac rhythm abnormalities observed includes atrioventricular (AV) blocks and premature ventricular contractions (PVC) (see Figure 3.3).

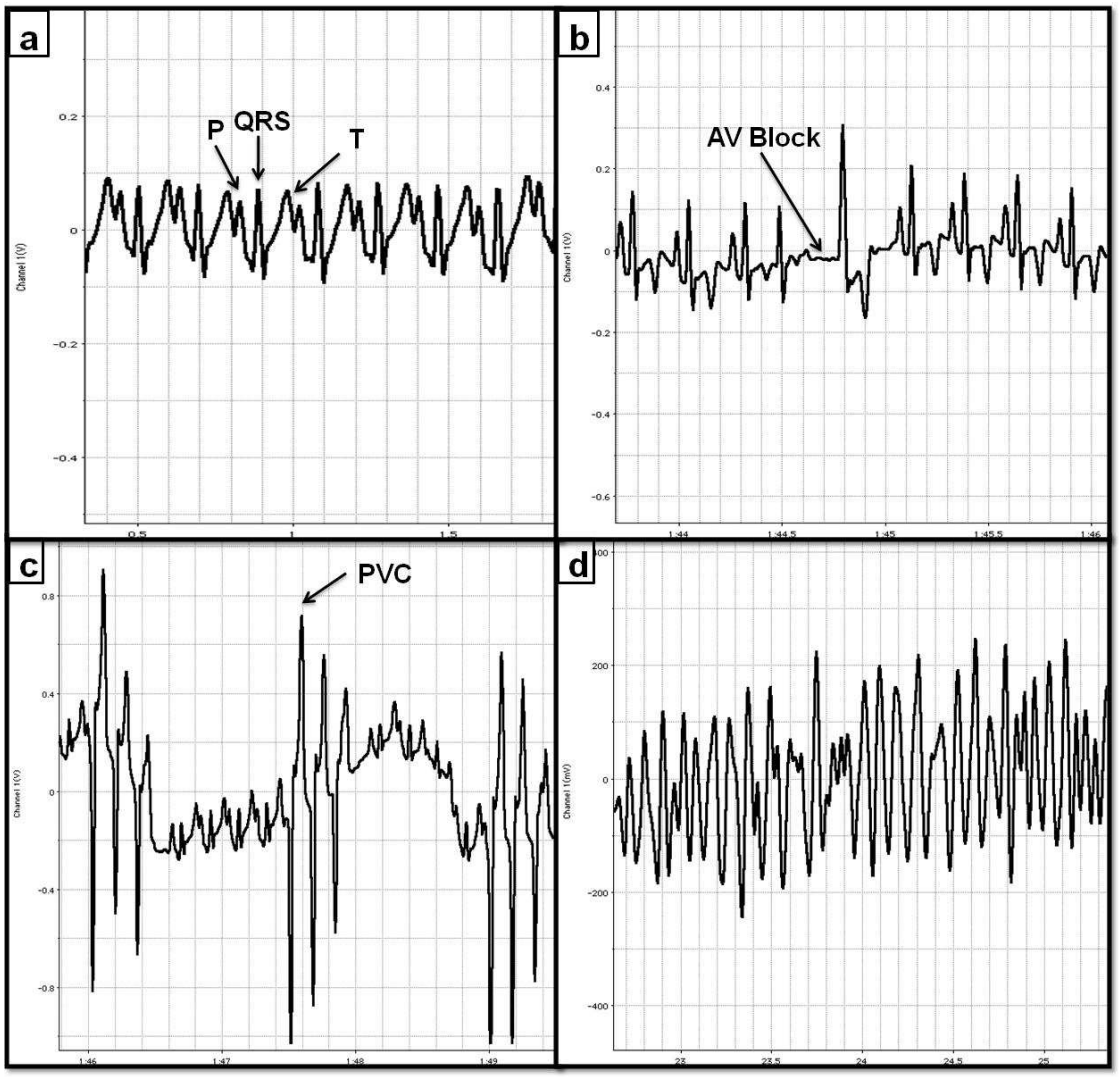

Figure 3.3. Representative electrocardiographic records from broilers showing normal and abnormal electrophysiological patterns.
a) Normal ECG, note clear recognizable P, QRS, and T wave characteristic of ECG pattern; **b)** AV Block, note the absence of QRS immediately after P wave; **c)** PVC, note three PVC in a row, Triplets pattern of PVC; **d)** Ventricular fibrillations, the ECG of the terminal electrical activity in the heart of moribund broilers shows waves characteristic of catastrophic arrhythmia, the wave pattern resembling torsade de points.

3.1.4. Blood Gas Measurements

For blood gas measurements about ten to fifteen percent of population was sampled at the end of the fifth week of the experiment. Approximately 0.5 mL blood sample was obtained anaerobically in a heparinized tuberculin syringe from the wing vein from randomly selected birds in each group. The samples were analyzed for pH, pCO_2, pO_2 and hemoglobin O_2 saturation percentage using a pH/Blood Gas Analyzer (Bayer Corporation, East Walpole, MA, USA).

3.1.5. Heart Tissue Procurement for Biochemical Analysis

Heart tissue samples used for biochemical analysis (high energy phosphate substrates, L-carnitine, and enzyme activity) were obtained from five randomly selected birds from each group [leghorns, broilers fed with restricted diet, broilers fed *ad libitum* diet (from group fed with control diet and from group fed with tested dietary ingredients) and broilers that developed CHF] at the end of the 6^{th} week of the experiment. Following cervical dislocation hearts were removed immediately and snap frozen in liquid nitrogen. The samples were stored in liquid nitrogen until analyzed.

3.1.5.1. High Energy Phosphates Estimation

A rapid High Performance Liquid Chromatography (HPLC) method was developed, validated and utilized for analysis of energy parameters, such as creatine phosphate (CrP), creatine (Cr), adenine tri-phosphate (ATP), adenine di-phosphate (ADP) and adenine monophosphate (AMP) simultaneously.

Analysis for above mentioned analytes was performed for samples from the mid portion of the left ventricular myocardium using the HPLC method as described in Olkowski *et al.* (2007a). Briefly, frozen samples were homogenized with 0.7 M cold perchloric acid in a ratio of 1 mL/100 mg tissue in pre-cooled tubes under low temperature. This homogenate was centrifuged at $12000 \times g$ for 5 minutes to remove precipitated protein. The suspension was neutralized with 0.25M potassium hydroxide to bring pH near 7. The final analysis was performed on an Agilent 1100 series HPLC

system (Hewlett Packard, Germany) using silica based reverse phase 3µm Luna C-18 column (Phenomenex, Torrance, CA), at detection wavelength of 210 nm. The analysis was performed using gradient elution in which column was first equilibrated with 20 mM of phosphate buffer (pH=7), then 6.5 minutes following sample injection 100% methanol was run on the column up to 12.5 minutes followed by phosphate buffer for an additional 5.5 minutes. The responses were evaluated by using Agilent chromatography software. Standards (mixture of CrP, Cr, ATP, ADP and AMP) were prepared from a stock solution. The method was developed and validated for selectivity, precision, accuracy and analyte recovery as follows.

3.1.5.1.1. Method Selectivity and Specificity

Analytes were identified on the chromatogram using standards for CrP, Cr, ATP, ADP and AMP (Sigma-Aldrich, St Louis, MO, USA) separately, followed by mixing them and running them together. Heart tissue samples were used to perform the validation phase. Peak purity for each analyte was established. Chromatogram was assessed for interferences from endogenous substances present in samples to ensure that the method is specific for the analyte of interest and no background interference from the matrix components was observed.

3.1.5.1.2. Calibration

Calibration curves were prepared by injecting a group of six standards; the dilution was made in such a way that the expected concentration of each analyte falls in the mid of calibration curve. The standard solutions were prepared by serial dilutions of CrP, Cr, ATP, ADP and AMP (Sigma St Louis, MO, USA) [with highest concentration being 80, 160, 160, 80 and 40 µg respectively per mL of phosphate buffer (20 mM)]. The coefficient of determination for each analyte was approaching 0.999 for standard curve.

3.1.5.1.3. Limit of Detection and Quantification (LOD, LOQ)

Limit of quantification (LOQ) refers to the lowest concentration of a compound that can be measured with acceptable precision and accuracy. Limit of detection (LOD) is the lowest concentration of our analyte that can be detected on chromatogram. LOD was interpreted by signal to noise ratio of 3:1; the LOQ with signal to noise ratio of 10:1. The limit of detection for each of our analyte was established before starting our real analysis by diluting our standard sample up to the limit at which it can be detected. There was no problem in detecting any analyte in our sample as all these energy parameters were much above the LOQ.

3.1.5.1.4. Precision

Precision is related to reproducibility or closeness of repeated measurements and can be expressed in terms of coefficient of variation (CV). This procedure showed a high degree of precision and reproducibility. The instrument precision was checked by repeatedly injecting the same sample thrice to know error due to instrument. The CV for CrP, Cr and AMP was lower than 1% (0.28, 0.09 and 0.46) except for ATP and ADP (2.15 and 1.26). Additionally, inter-assay precision was also checked by preparing three samples independently from the same tissue with intra-assay CVs for CrP, Cr, ATP, ADP and AMP of 6.62, 14.46, 18.57, 4.19 and 5.50%, respectively.

3.1.5.1.5. Analyte Recovery

Analyte recovery studies were conducted by running post extraction of myocardial samples to check for recovery. These recovery studies were performed in triplicate by repeatedly homogenizing, centrifuging and running the supernatant on HPLC. After the first extraction procedure, the recovery for CrP, Cr, ATP and ADP approached 100%, while for AMP the first extraction yielded only 95.38%. The subsequent extraction of the processed samples did not yield any measurable amount of the analytes except for AMP, where it yielded all the remaining analyte present in sample after first extraction.

3.1.5.1.6. Stability of Analyte in Storage

Stability of our standard solutions stored under different storage conditions was investigated. No significant decrease in levels of these analytes stored at -20º C was observed over a period of three days.

3.1.5.1.7. Accuracy

Accuracy is the closeness of the measured value to the true value of the analyte. This was measured by analyzing a sample (reference material) in triplicate with known concentration of respective high energy phosphate compounds and by comparing this measured value with true value. From accuracy studies, it was observed that our estimation was in close agreement with the true values i.e. the method overestimated CrP by 3.39%, and underestimated Cr, ATP, ADP and AMP by 0.42, 1.74, 1.48 and 0.83%, respectively.

3.1.5.1.8. Critical Evaluation of Methodology

This method is highly suitable for analyzing the high energy phosphates together on a single chromatogram. During the whole procedure temperature was kept as low as possible to avoid any loss of CrP, ATP and ADP from the myocardium. In addition special attention was given in the determination of CrP because this analyte was eluted near the void volume such that any un-retained component could affect our results, but no such interference was observed.

3.1.5.2. Enzyme Activity Assays

We focused on two cytosolic [Creatine Kinase (CK) and Lactate Dehydrogenase (LDH)] and two mitochondrial [Pyruvate Dehydrogenase (PDH) and alpha-Ketoglutarate Dehydrogenase (α-KGDH)] enzymes involved in energy synthesis and transformation pathways.

The activities of these enzymes were measured in heart tissue obtained from the mid portion of the left ventricle free wall using five samples in each group. Briefly, aliquots of 300 mg of frozen samples were homogenized in phosphate buffer (50 mM, pH 7.4) in the ratio of 100 mg tissue per 1 mL buffer in test tubes pre-cooled with ice. The homogenate was centrifuged at 2,500×g for 10 min at 4°C and the suspension was further centrifuged at 12,000×g for 10 min. The supernatant was used for CK and LDH measurements. The CK activity was measured using a CK kit (Roche Diagnostics, Indianapolis, IN, USA) while LDH was measured using LD-L10 kit (Sigma Diagnostics Inc., St. Louis, MO, USA). The mitochondria containing pellet was further washed three times to remove remnants of cytosolic enzymes. This pellet was re-suspended in Tris buffer (50 mM, pH 7.6 with 0.5% Triton).

PDH and α-KGDH activities were assayed in mitochondrial extracts. PDH activity was measured as described by Chiang and Sacktor (1975) with minor modifications where the final components of the incubation media were 2 mM pyruvate, 2.5 mM NAD, 0.15 mM flavin adenine dinucleotide, 2 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.13 mM Coenzyme A, 2.6 mM dithiothreitol, and 30 mM Tris buffer at pH 7.2. The α-KGDH activity was measured in principle as described before (Olkowski and Classen, 1999) with modifications, where the final components of the incubation media were 3.2 mM α-keto glutaric acid, 2 mM NAD, 0.5 mM Coenzyme A, 0.7 mM thiamine pyrophosphate, and 1 mM $MgCl_2$.

The reaction was initiated by adding 20 µL of cytosolic or mitochondrial fraction in 200 µL of cocktail per well in a 96 well micro plate pre incubated at 37°C for all these enzymes. Enzyme activity measurements were performed at 340 nm using a microplate reader SpectraMax Plus (Molecular Devices, CA, USA). The final activity measurements were performed during the linear phase of responses pre-established during validation phase. These measurements were performed in a single assay for each enzyme to avoid inter assay variability.

3.1.5.2.1. Method Development and Validation

During method development, the responses from enzyme assays (optical density: OD) were plotted with respect to time of reaction and protein content to determine the time and amount of protein content beyond which reactions were no longer linear (i.e. due to possibly factors such as substrate depletion) (Figure 3.4).

The assays were validated for the linearity of responses for time of reaction and protein content. The linearity of responses for time and protein content course were used to determine the time and amount of protein required; those values that fell within the middle of the trend lines (Figure 3.5 and 3.6) were used for final activity measurements.

The PDH and α-KGDH assays were assessed for dependence on various cofactors present in the reaction mixture (Table 3.2). The reaction for both enzymes was found to be 100% in the presence of all the added cofactors.

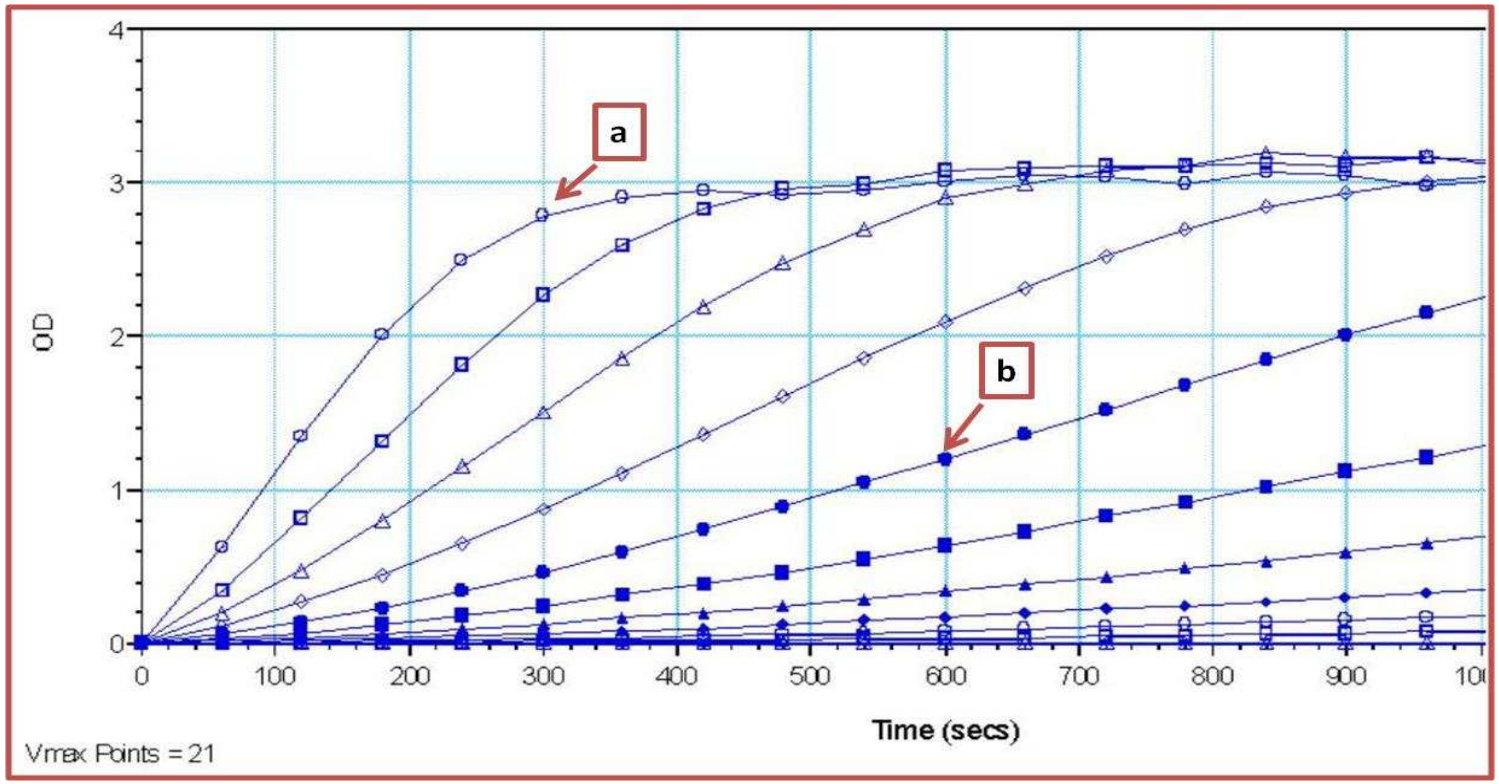

Figure 3.4. The responses of protein content at different time interval for creatine kinase.
At higher protein content (0.44mg/ mL) (a) substrate get exhausted earlier and there is no further activity, while below a particular protein content (0.05mg/ mL) (b) reaction keeps on going for a long period of time.

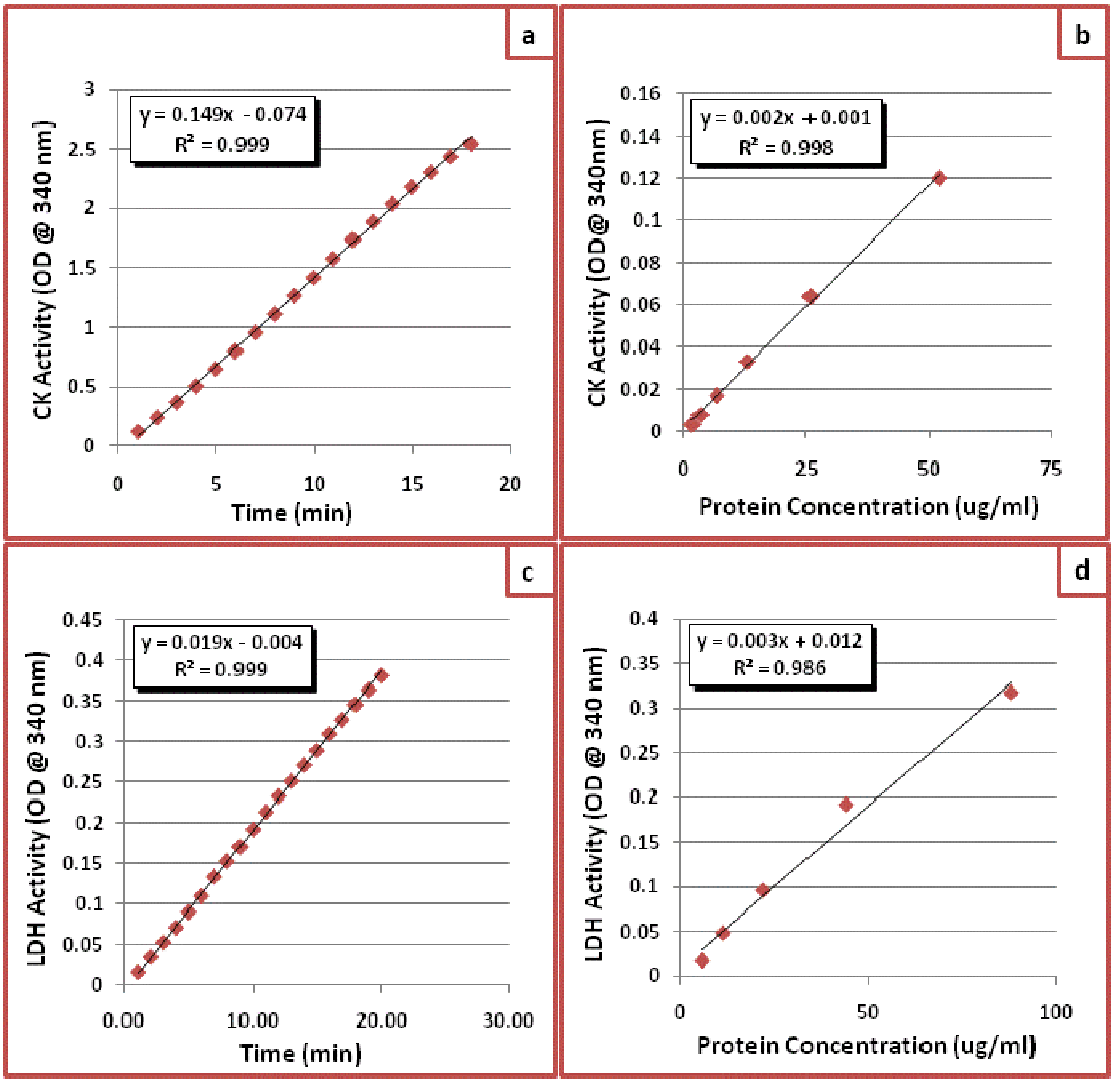

Figure 3.5. The linearity of responses for time and protein content for creatine kinase (CK) and lactate dehydrogenase (LDH) enzymes in heart tissue.
a & c) Activity (optical density; OD measured at 340nm) as a function of time; b & d) Activity (OD measured at 340nm) as a function of protein concentration. Note, the coefficient of determination approaches 0.99 for all enzymes. The time and protein content used for final enzyme assay conditions was chosen from the mid portion of the activity as a function of time/protein concentration course.

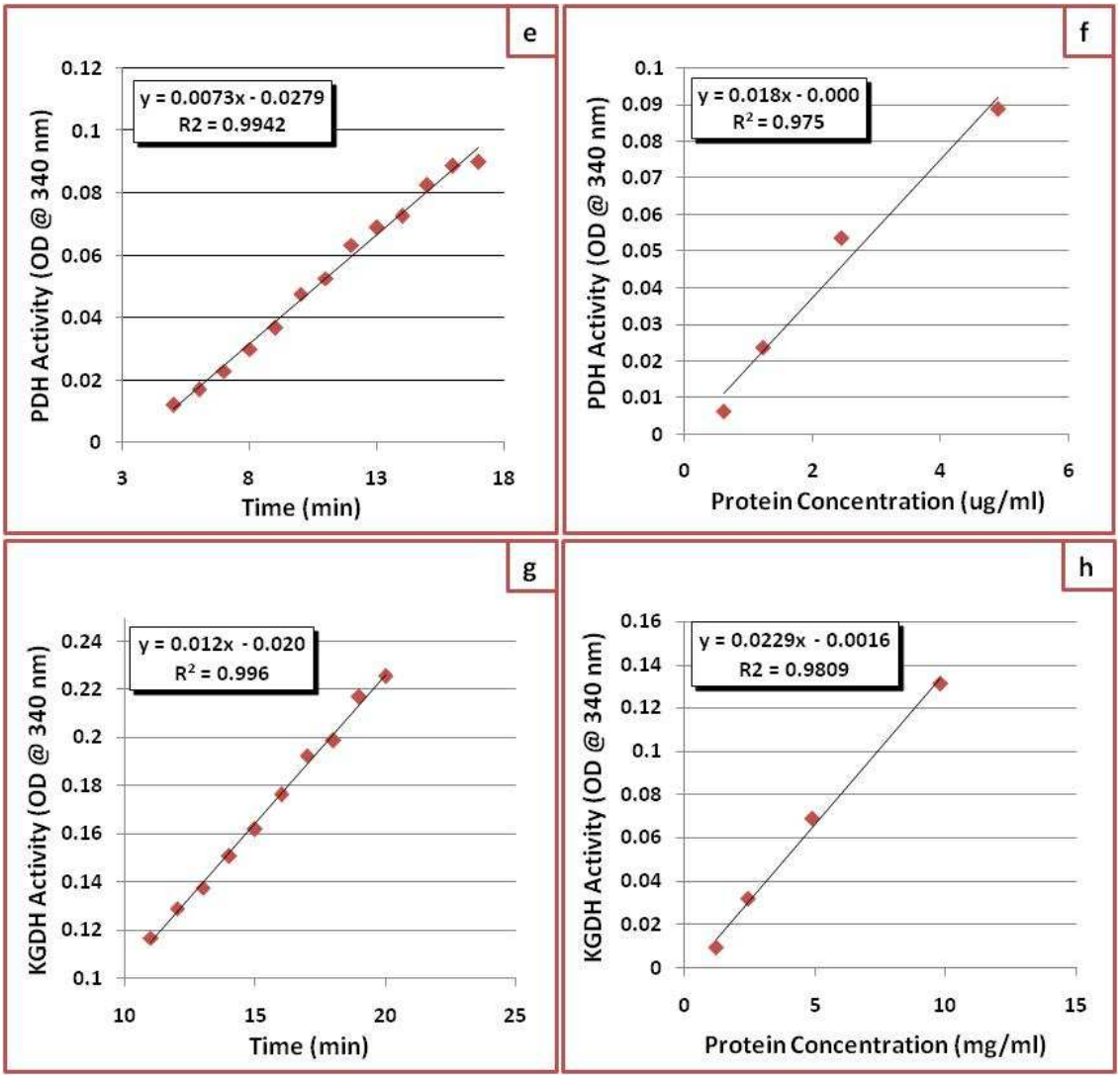

Figure 3.6. The linearity of responses for time and protein content for pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (α-KGDH) enzymes in heart tissue.
e & g) Activity (optical density; OD measured at 340nm) as a function of time; **f & h)** Activity (OD measured at 340nm) as a function of protein concentration. Note, the coefficient of determination approaches 0.99 for all enzymes. The time and protein content used for final enzyme assay conditions was chosen from the mid portion of the activity as a function of time/protein concentration curves.

Table 3.2. Cofactor requirements for pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (α-KGDH) activity.

Reaction Mixture	**Heart KGDH (% Activity)**
Complete Reaction	100%
Omitted: ketoglutarate	ND
Omitted: NAD	ND
Omitted: Coenzyme A	35%
Omitted: TPP	65%
Reaction Mixture	**Heart PDH (% Activity)**
Complete Reaction	100%
Omitted: Pyruvate	ND
Omitted: NAD	ND
Omitted: Coenzyme A	60%
Omitted: FAD	49%
Omitted: TPP	26%

ND: not detectable

3.1.5.3. Enzyme Sensitivity to Oxidative Stress

Activity of selected cytosolic (CK and LDH) and mitochondrial (PDH and KGDH) enzymes were tested using an *in vitro* indicator of oxidative stress. The activity of these enzymes in heart tissue obtained from the mid portion of the left ventricle free wall of fast-growing *ad libitum* fed broilers was tested by using either H_2O_2 or tertiary butyl hydroperoxide. The components of cocktail mixture were the same as described previously for each enzyme with the exception of presence of indicator of oxidative stress i.e. either H_2O_2 or tertiary butyl hydroperoxide (TBH). The activities of all the above mentioned enzymes were tested in presence of serial dilutions of H_2O_2 (195.6 mM). In addition the activities of PDH and KGDH were also assessed in the presence of serial dilutions of TBH (231.3 mM). Before conducting the final measurements each component of cocktail was tested in presence of H_2O_2 or TBH and it was established that cocktail components of these enzymes were not affected by the presence of H_2O_2 or TBH.

The reaction was started by adding 20 µL of cytosolic or mitochondrial fraction in 200 µL of cocktail plus 5 µL of serially diluted H_2O_2 or TBH per well in a 96 well micro plate pre incubated at 37°C. Enzyme activity measurements were performed at 340 nm using a microplate reader SpectraMax Plus (Molecular Devices, CA, USA). The final activity measurements were performed during the linear phase of responses pre-established during validation phase as described previously.

3.1.6. Post Mortem Examination

Detailed gross post-mortem examination was performed on all mortalities, euthanized birds, and all remaining birds upon termination of the study. Diagnosis of sudden death syndrome (SDS) was made when death occurred in well grown, apparently normal birds, without any other cause of death evident upon post mortem examination. The diagnosis of congestive heart failure (CHF) was based on findings of gross dilation of the ventricular chambers along with accumulation of ascitic fluid in abdominal cavity.

At termination of the experiment all surviving birds were subjected to gross post mortem examination. The diagnosis of sub-clinical heart disease was made when broilers did not show overt signs of heart failure on routine clinical observation, but post mortem examination revealed gross changes such as ventricular dilation, extensive pericardial effusion, and changes on the edges of left atrioventricular (AV) valve. These sub-clinical cases were graded based on the severity of lesions. The pericardial effusions were graded based on the amount of fluid in the pericardial sac. A large volume (>5 mL) of pericardial effusion with severely distended pericardium was classified as severe lesion and was considered as a variable of clinical significance (Olkowski *et al.*, 2003b). The dilations of ventricular chambers were graded based on dilation of ventricular chambers and thinning of ventricular wall (for details see footnotes in Figure 3.7). Similarly, the damage to left atrioventricular valve were also graded based on the severity of lesions (for details see footnotes in Figure 3.8).

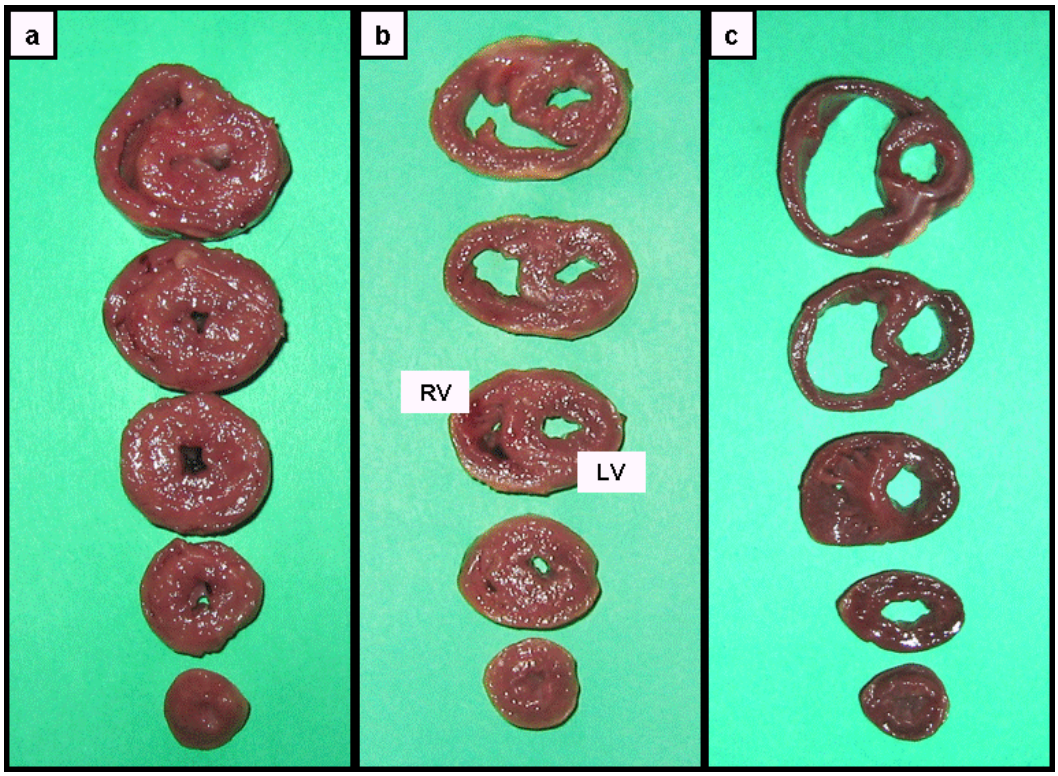

Figure 3.7. Serial sections of broiler heart representing morphological changes characterized as mild ventricular dilation (a), moderate ventricular dilation (b), and severe ventricular dilation (c).
RV indicates right ventricle, LV indicates left ventricle. Ventricular Dilation: a) Ventricles with mild dilation of chambers, revealing normal tone in ventricular wall along with small increase in diameter, dilation was not well appreciated from outside in intact heart but can be observed upon transverse cross section of heart. b) Ventricles with moderate dilation of chambers, dilation of ventricle was appreciated from outside in intact heart but lesser than severe dilation and the wall is moderately thin. c) Severely dilated ventricles with collapsed wall due to lack of tone and very thin ventricular wall, dilation was well appreciated in intact heart due to its increased diameter.

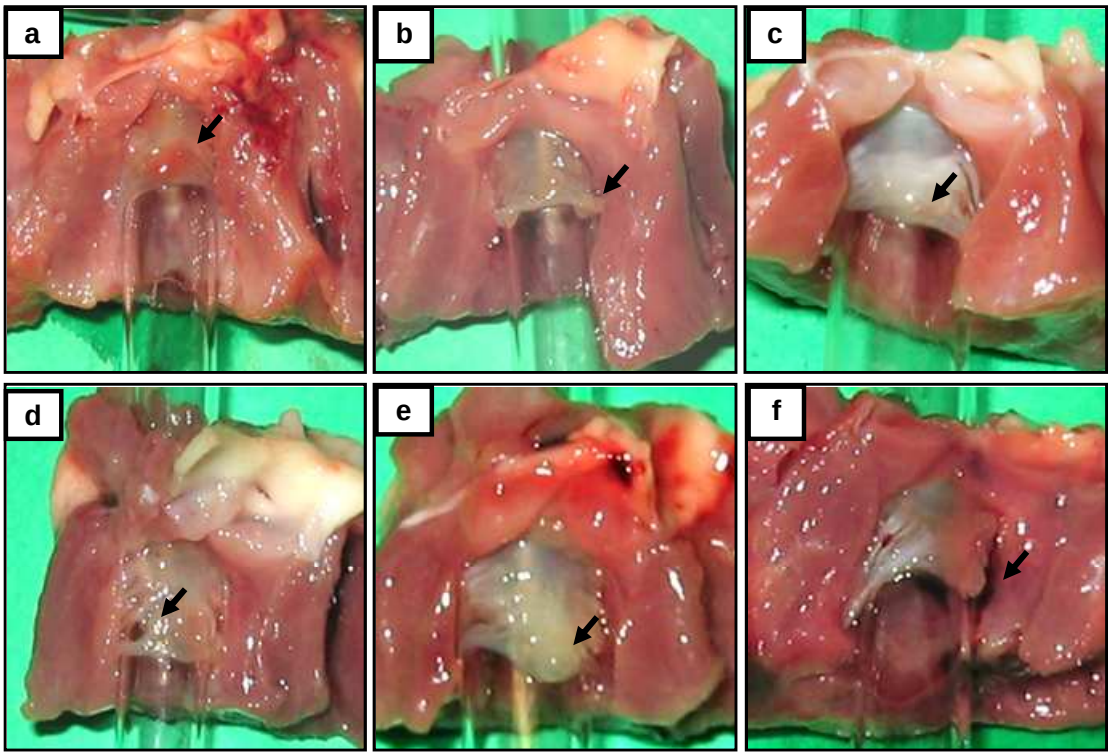

Figure 3.8. Broiler hearts representing morphological changes in left atrioventricular valve, characterized as mild degeneration (a), moderate degeneration (b & c), and severe degeneration (d, e & f).
Left atrio-ventricular valve damage: Lesions include nodular changes on the edges of the valves, chordae tendinae and around the annulus. **a):** Mild degeneration, note thickened leaflets with small nodules on edges of left AV valve (arrow). **b, c):** Moderate degeneration, note thickened and misshapen AV valves (arrow), a result of nodular changes. **d, e, f):** Severe degeneration, note thickened and grossly misshapen left AV valves (arrow), a result of nodular protrusion from the valves. In extremely damaged valves ruptured chordae tendinae can be observed.

3.1.7. Transmission Electron Microscopy (TEM)

To determine the ultrastructural changes associated with dietary treatments (vitamin A, D3, methanol soluble factors present in meat meal) hearts from three birds from each dietary treatment were processed for TEM. In addition, to explain pathogenesis for heart failure in broilers, additional heart samples were processed from

the group considered as resistant to heart failure (i.e. leghorns) and from birds that developed fulminant CHF. Heart tissue samples from the mid portion of the left ventricular wall were processed immediately after the birds were euthanized. The sections of myocardium approximately 5 mm in thickness were fixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer. After glutaraldehyde fixation, tissue sections were washed three times with 0.1 M cacodylate buffer and left overnight in this buffer. Subsequently, these tissue samples were fixed with 1% osmium tetroxide in 1.25% bicarbonate buffer (pH 7.2) for two hours at room temperature. After osmium fixation these samples were washed with 50% ethanol for 5 minutes, followed by one hour submersion in saturated uranyl acetate in 70% ethanol and subsequently dehydrated with ascending concentrations of ethanol. After dehydration, samples were washed three times with propylene oxide and embedded in epon/aradldite. Ultra thin sections were obtained using microtome on copper grids and stained with uranyl acetate and lead citrate. Electron micrographs were taken on a Philips 410 LS transmission electron microscope.

3.1.8. Scanning Electron Microscopy

Samples of posterior vena cava were fixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer, and were further processed as described previously by Olkowski *et al.* (2001). Briefly, fixed samples were washed with de-ionized water. These samples were subsequently dehydrated in graded concentrations of acetone and subsequently washed with 100% acetone three times each for 5 minute duration. The dehydrated samples were freeze dried and subsequently mounted on aluminum stubs with exposed longitudinal fractured surface and finally sputter coated with gold. The gold coated samples were examined under JEOL 840A scanning electron microscope.

3.1.9. Light Microscopy

The hearts from broilers fed the treated diet and blood vessels from apparently normal birds and birds with CHF were removed and processed for histopathological examination immediately after cervical dislocation. Following fixation in 10% buffered

formalin, blocks of myocardium were embedded in paraffin. Sections (5 μm thickness) were processed for light microscopy and stained with hematoxylin/eosin. The blood vessels were stained using haematoxylin/orcein/phyloxin/saffron (HOPS).

4. EXCESSIVE DIETARY VITAMIN D SUPPLEMENTATION AS A RISK FACTOR FOR SUDDEN DEATH SYNDROME IN FAST-GROWING COMMERCIAL BROILERS

4.1. Abstract

Broiler diets are frequently fortified with vitamin D_3 above the recommended level in an attempt to prevent commonly occurring leg problems. Since the basal levels of dietary vitamin D_3 are rarely known, there is a risk of over-supplementation. Over-supplementation of vitamin D_3 has been shown to have detrimental effects on the heart. Sudden death syndrome (SDS) is a condition commonly observed in broiler flocks and is associated with acute heart failure. The present study examines the effects of excessive levels of vitamin D_3 on cardiac health in fast-growing broiler chickens. Commercial male broilers (Ross X Ross 308) were exposed to either a commercial diet or a commercial diet supplemented with vitamin D_3. Throughout the trial all birds were monitored several times daily for overt signs of heart disease, and periodically electrocardiographic measurements were obtained. Morbidity and mortality data were collected daily. On day 32 a simulated stress challenge consisting of a single injection of epinephrine (100 μg/kg BW) was administered under continuous ECG monitoring. Broilers fed the vitamin D_3 enriched diet were 2.5 fold more likely to succumb to acute heart failure and die of SDS ($p<0.05$). Electrocardiographic examination showed a higher rate of cardiac arrhythmia in birds fed the vitamin D_3 enriched diet (22.6%), in comparison to those fed the control diet (11.8%). The stress challenge test revealed that broilers exposed to high dietary vitamin D_3 were more susceptible to ventricular arrhythmia. Our findings indicate that over-supplementation of vitamin D_3 increases the risk of SDS in broilers, and that the most likely mechanism is associated with increased susceptibility of the ventricular myocardium to arrhythmia.

4.2. Introduction

Fast-growing broilers are highly susceptible to Sudden Death Syndrome (SDS), where healthy, well grown individuals die suddenly from no discernible cause (for review see Olkowski and Classen, 1995). In modern broiler flocks SDS may cause significant economic losses (Maxwell and Robertson, 1998).

The act of death in broilers succumbing to SDS is associated with fatal cardiac arrhythmia, and ultimately the birds die of ventricular fibrillation or cardiac arrest (Olkowski and Classen, 1997; Nain *et al.*, unpublished). A variety of factors including nutrition, genetic, and environment have been linked to the incidence of SDS (for review see Olkowski and Classen, 1995), but the conditions that trigger the catastrophic arrhythmic event leading to sudden death remain unknown. Recent observations suggest that stress induced arrhythmia may be a significant contributing factor to SDS (Olkowski *et al.*, 2007b).

Previous research from our laboratory showed that many fast-growing broilers are predisposed to cardiac arrhythmia, and that birds with a history of more complex arrhythmia are at high risk of sudden death (Olkowski and Classen, 1997). Fast-growing broilers also suffer from a variety of leg problems, and therefore broiler diets are frequently supplemented with vitamin D_3 above the recommended levels for prevention. Since the basal levels of dietary vitamin D_3 in feed ingredients are rarely (if at all) known, there is a risk of over-supplementation. It has been demonstrated that high levels of vitamin D_3 cause a variety of metabolic and pathological lesions in the cardiomyocytes (Wrzolek, 1985; Takeo *et al.*, 1991; Wrzolkowa *et al.*, 1991; Walentynowicz and Wrzolkowa, 1995; Walentynowicz *et al.*, 2004). The detrimental effects of vitamin D_3 on cardiac function can be associated with morphological damage to the myocardium and/or biochemical effects via mobilization of body calcium. Because maintenance of cardiac Ca^{2+} homeostasis is critical to excitation-contraction coupling, changes may have a direct impact on heart electro-physiological stability (Lee *et al.*, 1988; Takeo *et al.*, 1991; Chavan *et al.*, 2007).

It is possible that intake of vitamin D_3 above requirement in fast-growing broilers that are already predisposed to cardiac arrhythmia, may further complicate pre-existing myocardial electrical sensitivity and precipitate fatal cardiac arrhythmia. In order to test this hypothesis, the present study was designed to examine whether increased intake of vitamin D_3 contributes to the risk of sudden death in fast-growing broilers. First, we investigated whether high dietary vitamin D_3 increases the incidence of cardiac arrhythmia and SDS. In the second instance we investigated whether vitamin D_3 increases the susceptibility of the heart to arrhythmia under simulated stress conditions.

4.3. Materials and Methods

4.3.1. General

Two experiments were conducted using commercial (Ross X Ross 308) male broilers. In each experiment, 209 and 182 day old broilers were randomly allocated to four pens in first experiment and six pens in second experiment, respectively. The chickens in respective control and treatment groups were offered either commercial broiler diet (5,000 IU vitamin D_3/kg) or commercial diet fortified with vitamin D_3 at level approximately sixteen times of the recommended levels (80,000 IU/kg) prepared by mixing the commercial diet with pure vitamin D_3 (DSM Nutritional Products, Canada).

All chickens were housed from day one in environmentally controlled (temperature and ventilation) room under constant light. Feed and water were provided *ad libitum*. During the first seven days the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 21°C by the end of the third week, and 17°C by the end of the fifth week. This pattern of lowered environmental temperature is routinely used in our laboratory experiments to force the birds to increase their metabolic rate, which results in increased burden on the cardiovascular system. Consequently, this approach is very effective in precipitating heart failure in broilers predisposed to heart conditions.

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee and the procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

4.3.2. Clinical Monitoring

All birds were monitored several times daily for overt signs of heart failure. Extra effort was made to examine five birds that died suddenly in the presence of the investigator. Birds that showed signs of death consistent with SDS (manifested as a loud squawk, sudden loss of balance, and wing flapping) in the presence of the investigator were immediately removed from the pen. The affected birds were connected to an ECG monitor within 15 to 30 seconds following the first signs of acute heart failure and monitored until cessation of heart electrical activity.

4.3.3. Electrocardiographic Measurements

The ECG measurements were obtained from 34 and 31 birds from control and treatment group, respectively, at 28 days of age, using needle electrodes (lead II arrangement) implanted subcutaneously after induction of light anaesthesia as described by Olkowski and Classen (1998a). The signals from the ECG monitor were digitized using an analog to digital data recording unit and software (Mac Lab and Scope 3.3: AD Instruments Pty Ltd, Castle Hill, Australia) and processed using a Macintosh computer. The ECG data were evaluated for normal and abnormal heart electrophysiological patterns.

4.3.4. Stress Challenge Test

The susceptibility of broilers to cardiac arrhythmia under simulated stress condition and their ability to recover from stress-induced arrhythmia were compared between broilers fed the control diet and those fed the diet supplemented with excess dietary vitamin $D_{3.}$ At the end of the 5^{th} week of the experiment, randomly selected 12 birds from each dietary group were subjected to a simulated stress challenge after

inducing anaesthesia as described above. Simulated stress condition was induced by injecting epinephrine (Erfa Canada Inc., Westmount QC, Canada). Epinephrine (1 mg/ml) was administered intravenously (wing vein) as a bolus at a rate of 100 μg/kg body weight under continuous ECG monitoring. The optimal dose of epinephrine and the route of administration were established on the basis of preliminary studies. The potential effect of injection with vehicle (isotonic saline) only was examined in previously in our laboratory, and it has been concluded that sham injection had no effect (Olkowski *et al.*, 2007b). The variables of interest for the purpose of this study included time of arrhythmia initiation, frequency of arrhythmic episodes, duration of arrhythmia and morphology.

4.3.5. Post Mortem Examination

Birds that died or were euthanized during the course of the study were subjected to detailed post mortem examination. A diagnosis of SDS was made based on unexpected death in well grown birds, without any other cause of death evident upon post mortem examination.

4.3.6. Light Microscopy

Since some events of SDS occurred in the presence of the investigator, we had the unique opportunity to examine changes in the cardiac tissue not obliterated by post-mortem artifacts. The hearts from two broilers fed the vitamin D_3 enriched diet that died suddenly were removed and processed for histopathological examination immediately after death. Following the fixation in formaldehyde buffer, blocks of myocardium were embedded in paraffin. Sections (5 μm), the first longitudinal taken midway through the left, septal and right myocardium, and the second at the point in immediate proximity of ventricular myocardium to atrial myocardium were processed for light microscopy and stained with Hematoxylin/eosin.

4.3.7. Statistical Analysis

Data were analyzed using the microcomputer package Number Cruncher Statistical System (Hintze, 1995). Stress challenge test data were analyzed using GLM ANOVA and the incidence of SDS and abnormal rhythm data were analyzed by using Fisher's exact test. SDS mortality was analyzed using data combined from experiments 1 and 2. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

4.4. Results

All broilers appeared normal on overt clinical examination prior to data collection. There were no significant differences in body mass between broilers fed the control diet (mean 2203g ± 191.0 SD) and broilers fed diet supplemented with vitamin D_3 (mean 2135g ± 168.1 SD).

Electrocardiographic evaluation revealed that 4 out of 34 (11.8%) of broilers fed the control diet, and 7 out of 31 (22.6%) of broilers fed the vitamin D_3 enriched diet showed ventricular arrhythmia. Cardiac rhythm abnormalities included episodes of atrial and ventricular arrhythmia, but for the most part premature ventricular contractions (PVC) were observed.

Sudden death in one control broiler and four broilers fed diet supplemented with vitamin D_3 occurred in the presence of the investigator, and the ECG recordings from the moribund broilers were obtained. All of these broilers showed an ECG pattern typical of acute heart failure (Figure 4.1).

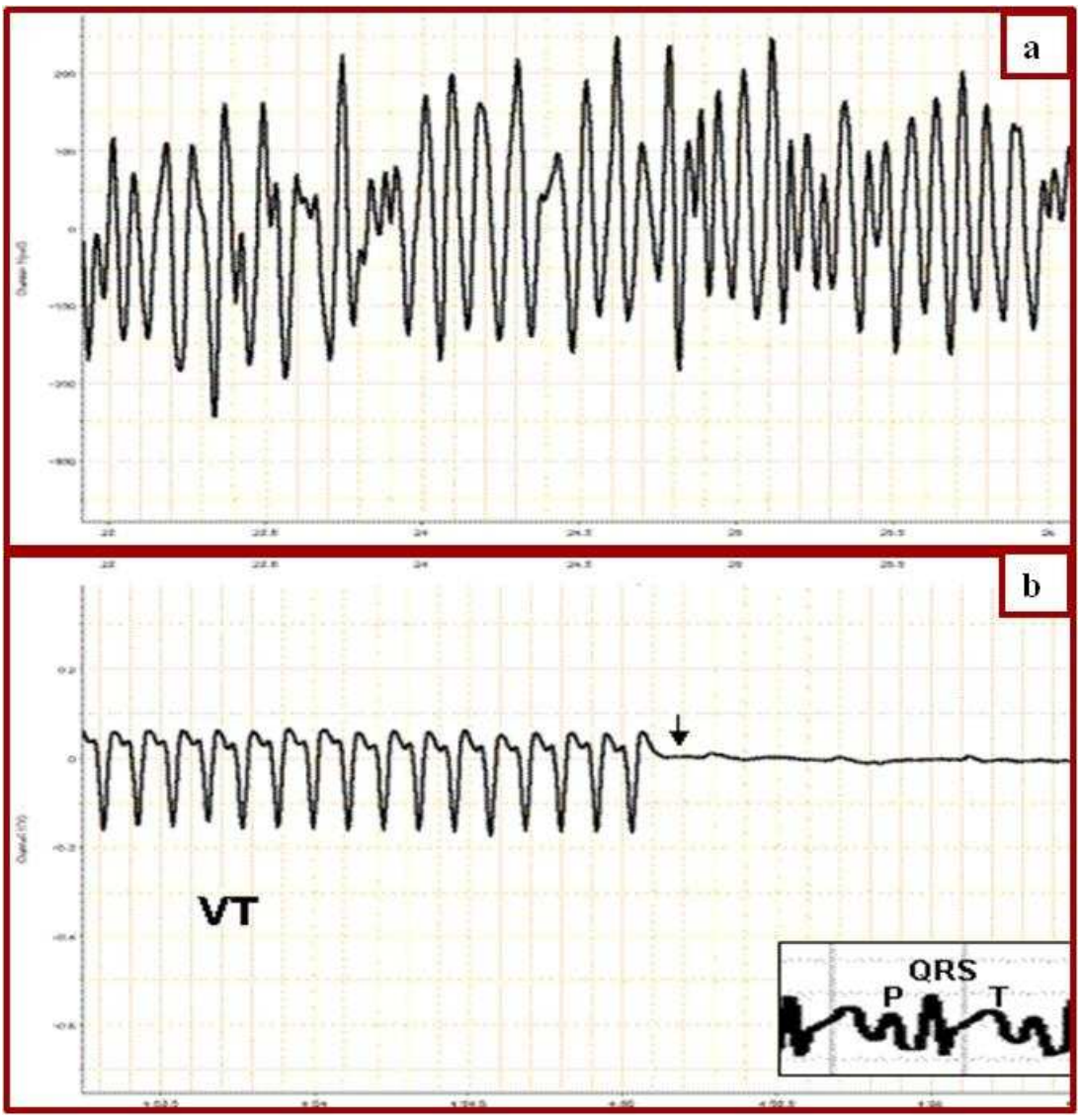

Figure 4.1. Electrocardiographic (ECG) records from two broilers fed the vitamin D_3 fortified diet that died of sudden death syndrome.
The ECG tracings of the terminal electrical activity in the heart of moribund broilers shows waves characteristic of catastrophic arrhythmia. In the first instance the ECG shows wave pattern resembling torsade de points and ventricular flutter (a), whereas in the second instance a pattern indicative of cardiac arrest (b). Notably, there are no recognizable wave forms P, QRS, and T characteristic of normal ECG pattern (Figure 4.1b, insert). The ECG recording shown in Figure 4.1b represents the initial ECG pattern typical of sustained ventricular tachycardia (VT) with 720 beats per minute, which subsequently changes abruptly (arrow) into an isoelectric line indicative of cessation of heart function.

The deaths of 26 broilers (8 in control and 18 in vitamin D_3 fortified group) in the present study were classified as SDS. Analysis of the relative risk showed that, in both experiments, broilers fed the vitamin D_3 enriched diet were 2.5 times more likely to die of SDS than those fed the control diet. Overall, broilers fed the vitamin D_3 enriched diet showed a significantly ($P<0.02$) higher incidence of SDS (Table 4.1).

Table 4.1. Incidence of sudden death mortalities in broilers fed the control diet and those fed the vitamin D_3 enriched diet.

	Experimental Group	**SDS Mortality**
Exp 4.1	Control (n=113)	† 6/113 (5.3%)
	Vitamin D_3 (n=96)	13/96 (13.5%) ‡
Exp 4.2	Control (n=91)	2/91 (2.2%)
	Vitamin D_3 (n=91)	5/91 (5.5%)
Exp 4.1 & 4.2 Combined Data	Control (n=204)	8/204 (3.92%)
	Vitamin D_3 (n=187)	18/187 (9.63%)
Significance (Fisher's Exact Test)		P<0.02

† Frequency of occurrence; ‡ % of examined

The responses to the simulated stress challenge with epinephrine were evaluated based on time elapsed from the challenge to first PVC, duration of arrhythmia, number of PVCs, and their morphology (Table 4.2). The arrhythmic episodes of PVCs in broilers fed the vitamin D_3 enriched diet tended to commence earlier, and lasted significantly longer than in broilers fed the control diet ($p<0.003$). The ventricular arrhythmias commonly observed were either bigeminy, trigeminy patterns of PVCs, or runs of ventricular tachycardia (five or more consecutive PVCs).

Table 4.2. Effects of vitamin D_3 enriched diet on cardiac susceptibility to stress induced arrhythmia. The effect of stress was simulated by injection of epinephrine administered intravenously (wing vein) as a bolus at a rate of 100 µg/kg body weight under continuous ECG monitoring.

	Stress Test Challenge Parameter Ventricular Arrhythmia				
	Time Variables (seconds)		**Frequency Variables (# of PVC episodes)**		
Treatment Group	**Initiation**	**Duration**	**0 to 5**	**>5**	**NSVT**
Control (n=12)	19 ±6.1	77 ±22.4	†7/12 (58%) ‡	5/12 (42%)	1/12 (8%)
Vitamin D_3 (n=12)	8 ±1.8	184 ±22.9	0/12 (0%)	12/12 (100%)	3/12 (25%)
Significance	*P = 0.09	*P < 0.003	**P < 0.002	**P = 0.02	**P = 0.29

NSVT=non sustained ventricular tachycardia (5 or more consecutive PVC)
Time values are means ± SE; † Frequency of occurrence; ‡ % of examined; *Analyses of Variance, **Fisher's Exact Test

Broilers showing less than five PVCs in response to simulated stress challenge were classified as at mild risk, and birds with more than five PVCs were considered to be at moderate to high risk of acute heart failure. Four broilers that were classified as being at high risk of life threatening arrhythmia predominantly showed runs of ventricular tachycardia.

The majority of responses to simulated stress in broilers fed the control diet can be characterized as low risk arrhythmia (less than five PVC episodes), whereas the responses in all broilers fed the vitamin D_3 enriched diet were classified as moderate to high risk (more than five PVC episodes), and these differences were statistically significant ($p<0.02$). Nonsustained ventricular tachycardia (five or more consecutive PVCs) occurred more frequently in birds fed the vitamin D_3 enriched diet (3/12) than in control broilers (1/12). The examples of arrhythmias observed during stress challenge are shown in Figure 4.2.

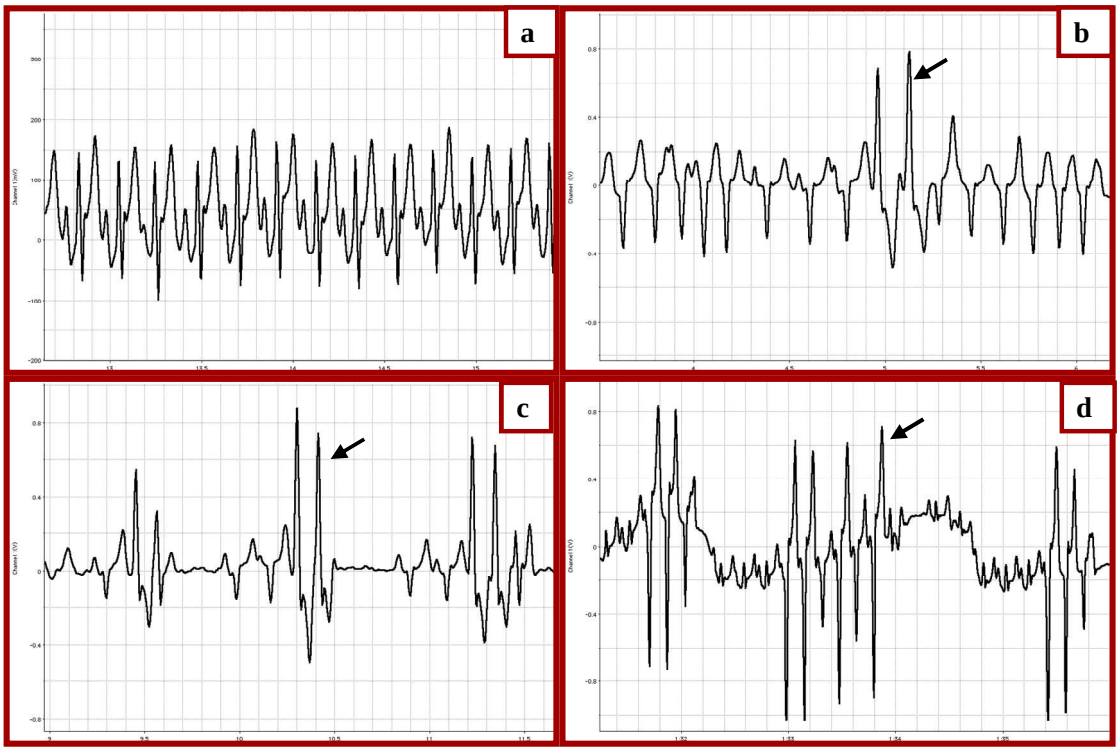

Figure 4.2. Electrocardiographic (ECG) records from broilers at various risk of life threatening arrhythmia identified during the simulated stress challenge induced by injection of epinephrine administered intravenously (wing vein) as a bolus at a rate of 100 µg/kg body weight.
The responses to simulated stress observed in the present study were characterized as: a) no changes in ECG pattern (Figure 4.2a), no risk of life threatening arrhythmia, b) sporadic ventricular arrhythmia consisting of single episodes of PVC (Figure 4.2b, arrow), low risk of life threatening arrhythmia, c) bigeminy or trigeminy patterns of PVCs (Figure 4.2c, arrow), moderate risk of life threatening arrhythmia, and d) multiple runs of 5 or more consecutive PVCs (Figure 4.2d, arrow), high risk of life threatening arrhythmia.

Histological examination revealed pathological changes in left and right ventricular myocardium located in cardiomyocytes and cardiac His-Purkinje conductive system (HPS). The intensity of the lesions can be described as focal, multifocal, and occasionally locally extensive (Figure 4. 3).

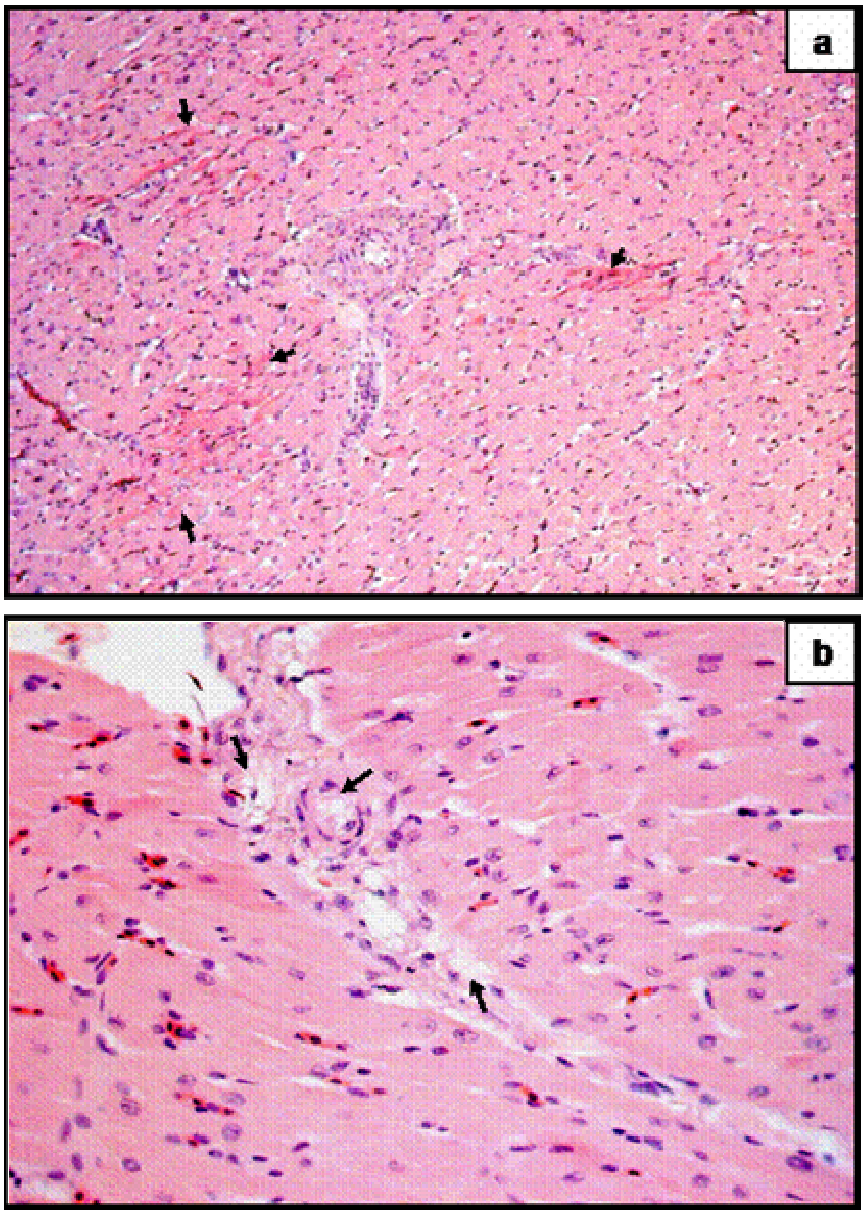

Figure 4.3. Histo-pathological features of the mural left ventricular myocardium in a broiler chicken from the group fed the high D3 diet and which died of sudden death syndrome (SDS).
(a) Extensive degenerative changes in the ventricular myocardium characterized by numerous cardiomyocytes with marked cytoplasmic eosinophilia and nuclear pyknosis (original magnification 100X). Noteworthy are locally extensive lesions characterized by distinct dull dark pink appearance of affected cardiomyocytes (arrows). (b) Pathological changes in the mural His-Purkinje system in the ventricular myocardium showing spongy and/or vacuolated sarcoplasm, eosinophilia, and nuclear pyknosis (arrows). The observed pathological changes in the cardiomyocytes and the conductive system are indicative that the affected cells are dead or in the process of dying.

4.5. Discussion

The findings from the present study indicate that high levels of dietary vitamin D_3 increase the risk of SDS in fast-growing broiler chickens. Given the fact that SDS is associated with lethal cardiac arrhythmia (Olkowski and Classen, 1997), it is most likely that there is a patho-physiological link between the higher incidence of complex, life threatening ventricular arrhythmia associated with high dietary vitamin D_3 and the risk of SDS.

Epinephrine, a hormone used in stress challenge test releases during acute stress, has been found to unmask the risk of arrhythmias in broilers (Olkowski *et al.,* 2007b). Interestingly, analysis of the physiological responses to simulated stress revealed that in contrast to normal broilers from control group, the characteristic responses of myocardium in normal birds exposed to excess vitamin D_3 included: 1) higher susceptibility to stress induced arrhythmias, 2) reaction to the stress challenge with complex arrhythmias, and 3) poor ability of the myocardium to recover from arrhythmic episodes. As such, these responses ought to be considered as factors increasing risk of SDS in susceptible broilers.

The higher susceptibility of broilers fed high levels of vitamin D_3 to cardiac rhythm disturbances, and the risk of SDS can be associated with primary factors that predispose myocardium to arrhythmia, and further by secondary factors that generate conducive milieu where an arrhythmic event may degenerate to catastrophic arrhythmia.

As a factor predisposing broiler's myocardium to arrhythmia, we have to consider the potential role of morphological changes seen in the hearts of SDS birds. Recently, we demonstrated that broilers succumbing to SDS show specific morphological changes in cardiomyocytes and His-Purkinje conductive system (HPS) (Olkowski *et al.,* 2007b). Interestingly, the changes seen in His-Purkinje fibers are clearly associated with apoptosis, which indicate significant physiological changes leading to premature deletion of these cells. Limited data from the present study

indicate that, in comparison to spontaneous cases of SDS (Olkowski *et al.*, 2007b), the morphological changes in the hearts of SDS birds from vitamin D_3 group are qualitatively similar, but they appear to be more extensive. Consistent with our findings, other workers demonstrated that excess dietary vitamin D_3 can cause heart lesions which include disruption of the structural integrity of myocardium with fragmentation of the cardiac muscle bundles and a disruption of extra-cellular matrix (Walentynowicz and Wrzolkowa, 1995; Lee and Lee, 1998; Walentynowicz *et al.*, 2004).

The morphological changes in the cardiomyocytes and HPS are undoubtedly of major significance as factors predisposing broiler myocardium to arrhythmia. Dying cardiomyocytes go through a period of increased excitability (Nerheim *et al.*, 2001), and lesions in heart muscle increase the risk of arrhythmic activity, and may lead to sudden cardiac death (for review see Swynghedauw, 1999). Hence, the morphological changes in the HPS seen in broilers may be considered as a possible trigger of ventricular arrhythmia (Berenfeld *et al.*, 1998; Lopera *et al.*, 2004), and consequently the primary cause of fatal arrhythmia and sudden death (Haissaguerre *et al.*, 2002).

However, the magnitude of the morphological changes alone is not likely of critical importance. It is possible that even mild, otherwise physiologically inconsequential morphological changes in the myocardium may be a trigger of life threatening arrhythmia in the situation when the electrical stability of the myocardium is compromised by some physiological or biochemical events. Therefore, an important mechanism that must be considered in order to explain why hypervitaminosis D_3 increased susceptibility of broiler myocardium to arrhythmia is the potential biochemical effect of vitamin D_3 disturbing the balance of electrolytes in heart tissue. It has been shown that oral administration of high doses of vitamin D_3 results in a marked increase in the myocardial calcium contents (Takeo *et al.*, 1991). An intracellular Ca^{2+} overload in the myocardium may play an important role in the genesis of arrhythmias by destabilizing the electrical and mechanical stability of the myocardium (Lee *et al.*, 1988). Scheideler *et al.* (1995) reported that slight deviations in dietary calcium and

phosphorus levels beyond recommendations can increase susceptibility to SDS mortality. Hence, the combination of dietary calcium and phosphorus imbalance with excessive vitamin D_3 supplementation may require additional consideration in the pathogenesis of SDS.

In the context of our findings, increased incidence of SDS in broilers fed vitamin D_3 should be considered from the interaction of two possible factors: 1) the presence of morphological changes being a factor conducive to arrhythmogenesis, and 2) electro-physiological changes in cardiac tissue associated with vitamin D_3 as a factor that may readily trigger fatal arrhythmia in the myocardium that is already at higher risk of electro-physiological instability associated with morphological changes.

The present study demonstrated that vitamin D_3 over supplementation in broilers resulted in a higher incidence of SDS in broilers. However, it is noteworthy that the stress challenge test revealed that broilers from the vitamin D_3 supplemented group were at higher risk of developing life threatening arrhythmia. Given the fact that commercial broilers are frequently subjected to various forms of environmental stress (e.g.overcrowding, antagonistic behavior, procedural and/or routine management activities) it is of particular interest to consider our overall finding in the context of stress. Indeed, stressful events may be an important trigger of fatal arrhythmia.

Ventricular arrythmogenic activity may be enhanced during exposure to stressful stimuli (Lown *et al.,* 1973; Kirby *et al.,* 1991; Sgoifo *et al.,* 1994), and stress has been shown to trigger fatal ventricular arrhythmia and sudden cardiac death (Sgoifo *et al.,* 1999; Lampert *et al.,* 2002; Rubart and Zipes, 2005). Many fast-growing broiler chickens are predisposed to cardiac arrhythmia and SDS (Grashorn, 1994; Olkowski *et al.,* 1997; Olkowski and Classen, 1998a; Korte *et al.,* 1999). In view of the present findings, it is possible that high dietary vitamin D_3 may further sensitize the broiler's myocardium to the effect of stress, and this may increase the risk of SDS whereby even mild arrhythmic episodes may degenerate to life threatening arrhythmia.

Our findings provide proof of principle that over-supplementation of vitamin D_3 in broiler diets may increase the risk of life threatening cardiac arrhythmia and incidence of SDS. This should be taken into consideration when supplements for management of leg problems are used. Further research to establish safe maximum levels of dietary vitamin D_3 is warranted.

5. EXCESSIVE DIETARY VITAMIN A AND D_3 SUPPLEMENTATION AS A RISK FACTOR FOR CONGESTIVE HEART FAILURE IN FAST-GROWING COMMERCIAL BROILERS.

5.1. Abstract

Broiler diet is frequently fortified with vitamin A or vitamin D_3 (D_3) above the recommended levels for better productivity and to prevent commonly occurring leg problems. Over-supplementation with vitamin A or D_3 has been shown to have detrimental effects on the heart. In order to evaluate the risk of vitamin A or D_3 over-supplementation on the incidence of congestive heart failure (CHF) in fast-growing commercial broilers, we examined the effects of high dietary vitamin A and D_3 in commercial male broilers. Approximately, three hundred male chicks were randomly assigned to six pens and offered a commercial broiler diet (5,000 IU vitamin D_3/kg and 15000 IU vitamin A/kg) or commercial broiler diet fortified with vitamin D_3 (80,000 IU/kg) or vitamin A (240,000 IU/kg). The broilers were housed in floor pens in an environmentally controlled room. Feed and water were provided *ad libitum*. All birds were monitored several times a day for overt signs of heart conditions. Morbidity and mortality data were collected daily. At day 32 of experiment, blood gas analysis was performed from randomly selected birds in each treatment. At the end of experiment, heart and lung samples were collected and processed for microscopic and ultrastructural evaluation. The risk of CHF was higher ($P<0.05$) in broilers fed the vitamin A or D_3 enriched diet as compared to broilers fed with control diet, with the incidence of CHF being 55.8% in the control, 71.9% in D_3 and 73.3% in vitamin A fortified group. The blood gas analysis revealed marked hypoxemia and lower Hb O_2 saturation percentage in vitamin A or D_3 group as compared to control group. The proportion of broilers with cyanosis was higher ($P<0.05$) in vitamin A (63.0%) and D_3 (65.5%) fed group as compared to control group (40.2%). Overall, the severities of the gross, microscopic and ultrastructural lesions were more pronounced in the broilers fed the vitamin A or D_3

fortified diet as compared to broilers fed with control diet. The present findings indicate that over-supplementation of vitamin A or vitamin D_3 increases the risk of CHF in broilers.

5.2. Introduction

Intensive continuous selection has been practiced in broiler industry from last five decades for fast growth and better feed conversion efficiency. Subsequent to this genetic selection along with improved nutritional strategies, modern broilers rapidly accrue body mass twice in weight and in half time as compared to its counterpart used 50 years ago (Havenstein *et al.,* 2003). Simultaneous with this improved performance, modern broilers become more predisposed to leg or heart related problems. The morbidity or mortality due to congestive heart failure (CHF) or ascites is one of the major causes of economic losses in broiler industry (Maxwell and Robertson, 1997).

Over-supplementation of vitamin A and D_3 were found to be associated with a variety of cardiac and metabolic lesions, and adversely affect heart function leading to increased risk of acute or chronic heart failure in various species (Wrzolek, 1985; Colbert, 2002; Nain *et al.,* 2007).

Vitamin A metabolite i.e. retinoic acid is involved in regulating cardiac form and function during embryogenesis and later in the postnatal life (Millemann *et al.,* 2007). Recent findings showed that vitamin A over-supplementation or over-expression of retinoic acid receptor leads to dilated cardiomyopathy, cardiac malformations or congestive heart failure in many species (Colbert *et al.,* 1997; Mulder *et al.,* 2000; Millemann *et al.,* 2007).

Concurrently, vitamin D_3 over-supplementation has been linked with heart failure with various microscopic lesions in myocardium (Bonucci and Sadun,1973; Walentynowicz and Wrzolkowa, 1995; Walentynowicz *et al.,* 2004). The observed microscopic lesion includes disruption and fragmentation of the myofibrillar

components in cardiomyocytes along with degenerative changes in the mitochondria (Walentynowicz *et al.,* 2004).

In commercial broiler situation, it is a common practice that vitamins D_3 is supplemented to avoid leg problems. Additionally, there is a common belief that vitamins are safe and their supplementation will improve productivity, but generally it is poorly understood that there are physiological and metabolic limits for their beneficial effects. In susceptible broilers, there may be a narrow margin between physiological requirements and adverse effects. The present study was conducted to test the hypothesis that excess dietary levels of these vitamins in the diet may increase the risk of CHF in broilers.

5.3. Materials and Methods

5.3.1. Animals, Treatments and Management

The present study was conducted using 299 commercial (Ross X Ross 308) male broilers. The birds were randomly allocated to three experimental groups in six pens, 45 to 50 birds per pen. The chickens in respective control and treatment (vitamin A and vitamin D_3) groups were offered either commercial broiler diet (5,000 IU vitamin D_3 and 15,000 IU vitamin A/kg) or commercial broiler diet fortified with vitamin D_3 (80,000 IU/kg) or vitamin A (240,000IU/kg), prepared by mixing the commercial diet with pure vitamin D_3 and vitamin A (DSM Nutritional Products, Canada) at a level approximately sixteen times of the commercial recomendations. Feed and water were provided *ad libitum*. As this was a small experiment and to comply with the guideline of 'Guide to the Care and Use of Experimental Animals' (Canadian Council on Animal Care, 1993) i.e. minimal use of animals for experiment, we designed a lowered brooding temperature protocol. This temperature protocol was used to precipitate CHF in practically all broilers predisposed to heart failure. The details of temperature protocol, animal management and feeding regime have already been published (Olkowski *et al.,* 1999; Nain *et al.,* 2008b).

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee and the procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

5.3.2. Clinical Monitoring

The birds were monitored daily for overt signs of heart disease i.e. fatigue, exercise intolerance, tachypnea, cyanosis and ascites. Birds were diagnosed with CHF based on detailed clinical investigation for the above mentioned clinical signs and confirmed by postmortem findings. Bluish discoloration of combs and wattles had been defined as cyanosis, linked with impaired cardiac functions in broilers. In order to assess the proportion of birds with cardiac dysfunction, the number of birds showing cyanotic combs and wattles were counted in each treatment during the 5^{th} week of experiment. Additionally, these visual measurements were validated with blood gas parameters to make sure that birds considered as cyanotic have impaired heart functions.

5.3.3. Blood Gas Measurements

Blood gas measurements were obtained from ten randomly selected birds from each group (5 per pen) during the fifth week of the experiment. For blood gas measurements, approximately 0.5 mL blood samples were obtained anaerobically from the wing vein. The samples were analyzed for pH, pCO_2, pO_2, and hemoglobin O_2 saturation using a pH/Blood Gas Analyzer (Bayer Corporation, East Walpole, MA, USA).

5.3.4. Post Mortem Examination

Detailed gross post-mortem examination was performed on all mortalities and birds euthanized during the course of the study. The diagnosis of fulminant congestive heart failure (CHF) was based on gross dilation of ventricular chambers along with accumulation of ascitic fluid in abdominal cavity. At the termination of experiment, all

surviving birds were subjected to gross post mortem examination. The sub-clinical heart conditions were diagnosed when broilers did not show obvious signs of heart failure on routine clinical observation, but post mortem examination revealed gross changes such as atrial and ventricular dilation, left atrio-ventricular (AV) valve changes and extensive pericardial effusion. These cases were evaluated for presence of cardiac lesions such as left atrioventricular valve damage and the degree of pericardial effusion. The pericardial effusions were graded based on the amount of fluid in pericardial sac. Large volume (>5 mL) of pericardial effusion with severely distended pericardium was classified as severe lesion and has been considered as a variable of clinical significance (Olkowski *et al.,* 2003b). The damage to left AV valve was graded (mild, moderate and severe) as described earlier (Olkowski *et al.,* 1998) using a set of photographs (for detail see Figure 3.8).

5.3.5. Vitamin D Analysis

For vitamin D analysis, blood plasma samples were obtained from five randomly selected broilers from group fed with vitamin D_3 fortified diet and control diet at the end of sixth week of age. Vitamin D_3 and its metabolite 25(OH) D_3 was measured in blood plasma using HPLC as described previously (Olkowski *et al.,* 2003a).

5.3.6. Light and Transmission Electron Microscopy

The heart and lung from three randomly derived broilers from each group (control, vitamin A and D_3) were processed for microscopic examination immediately after cervical dislocation. After fixation in 10% buffered formalin, samples of heart and lung tissue mid way from the left ventricular myocardium and through the long axis of the lungs were collected and embedded in paraffin. Longitudinal and transverse sections (5 μm) were processed for light microscopy and stained with hematoxylin/eosin. For electron microscopy, heart tissue from three randomly derived apparently normal broilers from group fed with vitamin D_3 fortified diets and control

diets were fixed in glutaraldehyde, and further processed as described previously by Olkowski *et al.* (2001).

5.3.7. Statistical Analysis

Data were analyzed using the microcomputer package Number Cruncher Statistical System (Hintze, 1995). Blood gas and vitamin D analysis data were analyzed using GLM ANOVA. The mortality/morbidity, descriptive clinical and pathological data were analyzed by using Fisher's exact test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

5.4. Results

The incidence of CHF was higher ($P<0.05$) in vitamin A (73.3%) and D_3 fortified groups (71.9%) as compared to group fed with control diet (55.8%) (see Table 5.1). Measurement of the incidence of cyanosis at day 32 revealed that the proportion of broilers with cyanotic combs and wattles were higher ($P<0.05$) in the group fed the vitamin A (63.0%) and D_3 (65.5%) supplemented diet as compared to the control group (40.2%).

Table 5.1. Incidence of congestive heart failure (CHF) in broilers fed the control diet andthose fed the vitamin A and D_3 fortified diet.

Experimental Group	**Birds allocated**	**CHF (Mortality/Morbidity)**
Control	**n=113**	$63/113^{a}$ † (55.8%) ‡
Vitamin A	**n=90**	$66/90^{b}$ (73.3 %)
Vitamin D_3	**n=96**	$69/96^{b}$ (71.88%)
Significance (Fisher's Exact Test)		$p \leq 0.05$

† Frequency of occurrence; ‡ % of examined
Values with different superscripts are significantly different ($P< 0.05$)

The blood gas analysis on birds with cyanosis revealed higher pCO_2, lower pO_2 and hemoglobin O_2 saturation percentage as compared to apparently normal birds (Figure 3.2). The blood gas findings in broilers oversupplemented with vitamin A or D_3 were indicative of hypoxemia (see Table 5.2). The values for blood pO_2 (30.1 mmHg) and HbO_2 saturation (56.2 %) in the group fed vitamin D_3 fortified diet were lower ($p<0.05$) as compared to broilers fed the control diet (pO_2: 38.4 mmHg and HbO_2 saturation: 72.1 %). The hypoxemia (pO_2: 35.9 mmHg), hypercapnia (pCO_2: 46.0 mmHg) and lower HbO_2 saturation (66.0%) was apparent in broilers fed with vitamin A fortified diet as compared to control group but the differences were not statistically significant ($p>0.05$).

Table 5.2. Effect of high dietary vitamin A, vitamin D_3 on blood gas parameters (pCO_2, pO_2 and HbO_2 Saturation %) measured from randomly derived broilers from control, vitamin A and vitamin D_3 fortified group.

Groups	**pH**	**pCO_2 (mm Hg)**	**pO_2 (mm Hg)**	**Hb O_2 Saturation%**
Control	7.42 ± 0.017	43.7 ± 2.04	38.4 ±1.68^{b}	72.1 ± 2.78^{b}
Vitamin A	7.41 ± 0.022	46.0 ± 3.24	35.9 ± 2.59^{ab}	66.0 ± 5.50^{ab}
Vitamin D_3	7.41 ± 0.012	45.6 ± 2.51	30.1 ± 2.12^{a}	56.2 ± 5.11^{a}
P Value	P = 0.66	P = 0.77	P < 0.04	P < 0.05

^{a,b} columns with different superscripts are significantly different (P< 0.05). Values are means ± SE (n=10)

The levels of 25-hydroxycholecalciferol [25(OH) D_3] (191.8 ng/mL) and cholecalciferol (D_3) (7.8 ng/mL) in blood plasma were higher (P< 0.05) (Table 5.3) in vitamin D_3 fed broilers as compared to broilers fed with control diet [25(OH) D_3: 63.3 ng/mL and D_3: 5.1 ng/mL].

Table 5.3. 25 Hydroxy Cholecalciferol [25(OH) D_3] and Cholecalciferol (D_3) levels in blood plasma measured from randomly derived broilers from control and vitamin D_3 fortified group.

Groups	**25(OH) D_3 (ng/mL)**	**D_3 (ng/mL)**
Control	63.3 ± 11.29^{b}	5.1 ± 0.63^{b}
Vitamin D	191.8 ± 11.378^{a}	7.8 ± 0.95^{a}
P value	$P < 0.001$	$P < 0.05$

^{a,b} columns with different superscripts are significantly different ($P< 0.05$). Values are means ± SE (n=5)

Post mortem examination on broilers with CHF revealed gross dilation of the ventricular chambers, left atrioventricular valve damage and severe pericardial effusions, along with ascitic fluid in abdominal cavity. Findings from broilers with pathological signs of sub-clinical heart disease revealed that the occurrence of severe left AV valve damage was numerically higher in the broilers fed the diet fortified with vitamin D_3 (43% of examined i.e. 13 out of 30) or vitamin A (40% of examined i.e. 10 out of 25), as compared to the group fed with control diet (27% of examined i.e. 11 out of 41). Proportion of broilers with severe pericardial effusions (>5 mL) was higher ($p<0.05$) in group fed with vitamin D_3 fortified diet (63% of examined i.e. 17 out of 27) as compared to group fed the control diet (28% of examined i.e. 7 out of 25). In vitamin A fortified diet, severe pericardial effusion was observed in 45% of examined broilers (i.e. 10 out of 22).

Histological evaluation of lungs in broilers from all treatments revealed cartilaginous irregular nodular structures throughout the lung tissue (Figure 5.1). The average counts on these nodular structures from right lung cross section revealed 8 nodules in broilers fed with control diet, 7 in boilers fed with vitamin A fortified diet and 16 in broilers fed with vitamin D_3 fortified diet. Qualitatively, the nodular structures were larger in diameter in broilers fed the diet fortified with vitamin D_3 versus broilers fed the control diet.

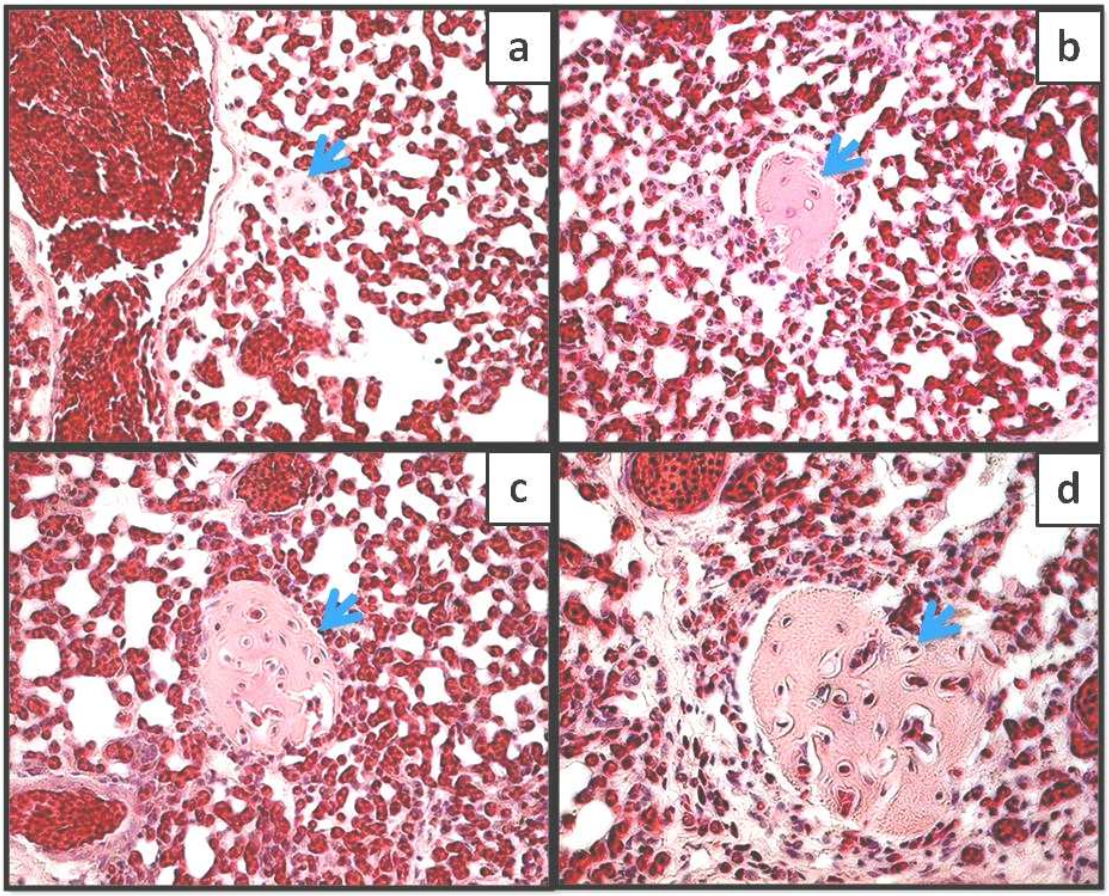

Figure 5.1. Sections of lung tissue from broilers showing nodular structures in para-bronchial air spaces.
Cartilaginous ectopic irregular nodular formations (blue arrows) in the para-bronchial air spaces, as size of these structures increases it reduces considerable portion of gas exchange area. Lung tissue with very small nodules (**a**) in initial stage of nodular formation, note; as size of this nodule increases (**b,c,d**) number of chondrocytes and mineralization increases.

Moreover, the histological evaluation of myocardium from all the broilers revealed cardiomyocyte degeneration irrespective of dietary treatment (Figure 5.2). The observed degenerative changes include sarcoplasmic eosinophilia, nuclear pyknosis, karyorrhexis and karyolysis. Comparatively, the frequency of these lesions were more abundant in broilers fed with vitamin A or D_3 as compared to broilers fed the control diet. The severity of lesions was most pronounced in broilers that developed CHF.

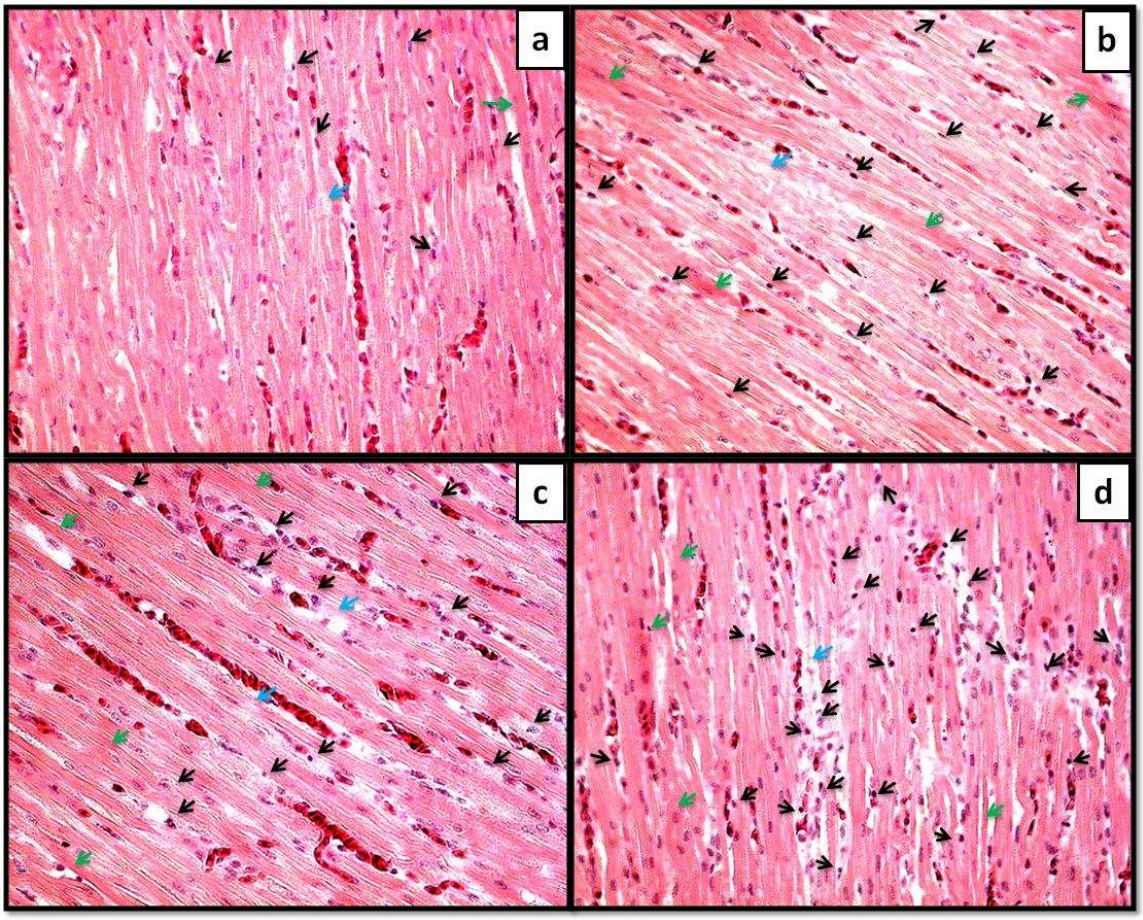

Figure 5.2. Representative histopathological sections from mural left ventricular myocardium of an apparently normal broiler fed with either the control diet (a), diet fortified with vitamin A (b) or vitamin D_3 (c) and from a broiler with congestive heart failure (d). Original magnification 400X.
The observed myocardial lesions include degenerative changes in sarcoplasm and nuclei of cardiomyocytes. Nuclear degeneration is observed in the form of nuclear pyknosis, karyorrhexis and karyolysis (black arrows). Additionally, in cardiomyocytes cytoplasmic eosinophilia (green arrow), an early indicator of cellular changes subsequent to myocardial injury can be observed. The lesions are most severe in a broiler with CHF. In some areas, a few cardiomyocytes appear wispy pale with reduction in typical banding pattern, indicative of loss and thinning of myofibrillar components (blue arrow), and in advanced cases cardiomyocyte dropout can be observed. Lesions are of similar qualitative features in all the three groups, but the degenerative changes were more extensive in broiler fed with vitamin A or D_3 fortified diet as compared to broiler fed with control diet, but of lesser magnitude as compared to a broiler with CHF.

The observed ultrastructural changes include myofibrillar dissolution and disintegration along with mitochondrial changes (Figure 5.3). Mitochondrial lesions include swelling, vacuolization, and disintegration of cristae. The myofibrillar disintegration and thinning of myofibrillar bundles were more apparent in broilers fed with vitamin D_3 fortified diet as compared to broilers fed with control diet.

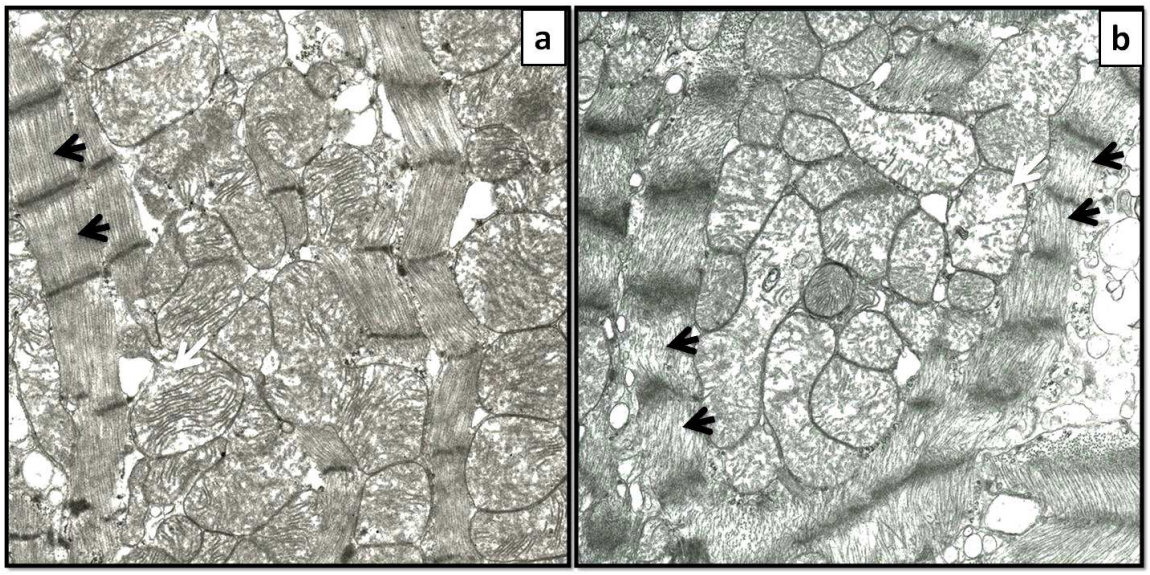

Figure 5.3. Representative electron micrograph of myocardium from a randomly derived broiler fed with control diet (a) or diet fortified with vitamin D3 (b) (Original magnification 4500X).
Careful evaluation of micrograph reveals myofibrillar thinning and disintegration in both broilers; however, comparatively this thinning of myofibrillar bundle (black arrow) is more apparent in vitamin D_3 fortified broiler as compared to control broiler. Similarly, mitochondrial swelling, vacuolization, destruction and pleomorphism (white arrow) can be observed in both broilers but more apparent in broiler fed with vitamin D_3 fortified diet.

5.5. Discussion

The findings from the present study indicate that excess of dietary vitamin A or D_3 increases the risk of CHF in fast-growing broilers. The presence of these vitamins in broiler diet directly increases the incidence of CHF in broilers. Further, supportive evidence was provided by gross, microscopic and ultrastructural findings, as lesions were more pronounced in broilers fed with vitamin A or D_3 fortified diet as compared to broilers fed with control diet. Hypoxemia and higher proportion of broilers with

cyanosis in group fed with vitamin A or D_3 fortified diet showed that these broilers were at increased risk of developing CHF. Taken together, all these findings indicate that vitamin A or D_3 used in broilers diet at the level used in present study are capable of inducing cardiac lesions and precipitate heart failure in susceptible broilers.

Ultrastructural findings in group fed with vitamin D_3 fortified diet were similar to those reported by other studies. Hypervitaminosis D_3 leads to loss of mitochondrial functions and myofibrillar protein components in cardiomyocytes of rats (Takeo *et al.*, 1991; Walentynowicz and Wrzolkowa, 1995). This myofibrillar loss has been found to be preceded by calcium deposition in cardiomyocytes (Wrzolkowa *et al.*, 1991). This damage to myofibrillar proteins is most probably by calcium activated peptidases (Walentynowicz and Wrzolkowa, 1995). Recently, we provided the proof that excess of vitamin D_3 in broilers diet increases the myocardial susceptibility to ventricular arrhythmia (Chapter 4). Hence, vitamin D_3 supplementation in broilers diet has adverse effect on cardiac function either through increased activity of peptidases or through increased susceptibility to arrhythmia.

Hypoxemia in broilers has been linked with a) poor heart performance (Olkowski and Classen, 1998b; Nain *et al.*, 2008b) b) cartilaginous ectopic irregular nodular formations in lungs, leading to elevated intrapulmonary shunt fraction (Olkowski *et al.*, 2005b). Increased nodular formations have been observed in broilers developing CHF. Hence, increased nodular formations along with increased cardiac lesion together leads to development of hypoxemia and ultimately higher susceptibility of broilers to development of CHF in group fed with vitamin D_3 fortified diet. This observed higher risk of CHF in broilers fed vitamin D_3 fortified diet is most likely associated with its metabolite i.e. 25(OH) D_3, as its concentration is about three fold in broilers fed vitamin D_3 fortified diet as compared to broilers on control diet.

Retinoic acid (RA), the active metabolite of vitamin A, by interacting with its retinoid receptors, regulates heart symmetry and morphogenesis (Chazaud *et al.*, 1999). Studies have established that vitamin A over-supplementation during early embryonic

development leads to cardiac malformations across various mammalian and avian species (Kraft and Juchau, 1993; Osmond *et al.,* 1991; Mulder *et al.,* 2000; Millemann *et al.,* 2007). Most of these studies have established the relationship between embryonic exposure of vitamin A with increased risk of cardiac dysfunctions, but none have established the relationship between vitamin A exposure during postnatal life and risk of heart failure. Colbert *et al.* (1997) demonstrated that retinoic acid receptor over expression leads to dilated cardiomyopathy and CHF in rats. Based on histo-pathological findings in the present study, we suggest that excess of vitamin A leads to increased risk of cardiomyocyte apoptosis and dysfunction and in the end stage to heart pump failure.

Interestingly, matrix metalloproteinase-2 (MMP-2), an enzyme involved in tissue remodeling, activity and expression has been found to be elevated in chicken chondrocyte culture treated with retinoic acid (Nie *et al.,* 1998). Ultrastructural and molecular changes in ventricular myocardium of broilers developing CHF has been found to be associated with increased activity and expression of MMP-2 (Olkowski *et al.,* 2003b; Yan *et al.,* 2008). Relatively high levels of MMP-2 activity have been observed in pericardial effusions from broilers with CHF (Olkowski *et al.,* 2003b). This evidence suggests that over expression of this enzyme might be another contributing factor leading to increased risk of CHF in broilers fed with vitamin A fortified diet.

Hence, it is reasonable to speculate that high incidence of CHF or ascites observed in some broiler flocks may be subsequent to over-supplementation of vitamin A or D_3 in diet. As these vitamins are supplemented in broilers diet for variety of reasons, but when the tolerance level in diet exceeded, then the risk of losses subsequent to heart failure may occur.

Our findings provide the proof of evidence that over-supplementation of vitamin A or D_3 in broilers diet at a level used in the present study increases the risk and incidence of CHF. As the supplementation of these vitamins is commonly practiced in

broilers diet, further research to establish safe levels of dietary vitamin A or D_3 is warranted.

6. PUTATIVE CARDIOTOXIC COMPOUNDS EXTRACTED FROM MEAT MEAL AS A POTENTIAL RISK FACTOR FOR THE DEVELOPMENT OF HEART FAILURE IN FAST-GROWING COMMERCIAL BROILERS

6.1. Abstract

Thermal processing of meat products generates a number of cardiotoxic compounds capable of inducing heart failure in both humans and laboratory animals. Such compounds may be present in broiler diets because supplements such as meat meal (MM) or fish meal (FM), which are commonly used in broiler rations, are rendered at high temperature. Our objectives were to evaluate whether the putative cardiotoxic compounds in MM increase the risk of heart failure in broilers. The treatment and control diets were prepared by mixing the condensed MM extract (equivalent to dietary MM inclusion of 25%) or placebo (condensed extraction medium) with commercial broiler feed, and the respective diets were offered to commercial male broilers randomly allocated to either treatment or control groups. Broilers fed a diet spiked with MM extract showed a higher incidence ($p<0.05$) of chronic heart failure (65.5%) in comparison to the control group (55.4%). Post mortem examination upon termination of the experiment revealed that, in comparison to control broilers, broilers fed diet containing MM extract showed higher incidence of lesions indicative of sub-clinical heart disease evidenced grossly by ventricular dilation and pericardial effusions, microscopically by changes characteristic of cardiomyocyte degeneration, and ultrastructurally by changes in contractile elements and in mitochondria. Measurements of cardiac high energy phosphates revealed that broilers fed the diet containing MM extract had lower ($p<0.05$) levels of cardiac energy reserve as compared to birds fed control diet. We conclude that cardiotoxic factors that can induce patho-physiological changes in the heart are present in MM.

6.2. Introduction

Selection for traits of economic value has resulted in a broiler genotype that has inherent predisposition to heart failure (Navarro *et al.*, 2006; Druyan *et al.*, 2007). Clinical observations and necropsy findings from several studies indicate that many commercial broilers show sub-clinical signs of heart condition, and are at higher risk of heart failure (Odom *et al.*, 1992; Owen *et al.*, 1995; Olkowski *et al.*, 1997; 1998; 1999; 2001; 2003b; Olkowski, 2007). However, factors that may increase the risk of heart failure in broilers are poorly understood.

Heart failure has been linked to cardiotoxic compounds that may be accumulated in animal tissue (Walker and Catron, 2000; Xie *et al.*, 2006). Recent research indicates that various cardiotoxic pollutants are present in fish (Hites *et al.*, 2004; Hamilton *et al.*, 2005). Furthermore, the etiology of heart failure in laboratory animals and humans has been found to be associated with cardiotoxic compounds generated during thermal meat processing (Davis *et al.*, 1994; Gaubatz, 1997; Dubuisson *et al.*, 2001).

Animal by-products such as MM and FM used as dietary supplements in poultry are processed at high temperature during the rendering process, there is little doubt that cardiotoxic compounds known to be generated during thermal processing of meat (Davis *et al.*, 1994; Gaubatz, 1997; Dubuisson *et al.*, 2001) are present in the MM or FM. Also, some plant protein supplements (e.g. soy bean meal) commonly used in broiler diets, are subjected to heat treatment to neutralize anti-nutritional factors, thus similar compounds may be derived from plant protein supplements (Yoshida *et al.*, 1978; Lan *et al.*, 2004).

Supplements of animal origin such as MM and FM that are commonly used in broiler diets may contain factors that are detrimental to cardiac health. Given the fact that many fast-growing broilers are already predisposed to heart disease (Navarro *et al.*, 2006; Druyan *et al.*, 2007, Olkowski, 2007), it is possible that cardiotoxic compounds present in broiler diet can further increase the risk of heart failure in susceptible

individuals. In order to test this hypothesis, the present study was designed to examine whether MM derived factors contribute to the risk of acute or chronic heart problems in fast-growing broilers. First, we investigated whether acidified methanol extractable factors from MM in diet increase the incidence of acute or chronic heart failure. In the second instance we investigated the effect of these factors on the basic parameters of heart performance, myocardial susceptibility to arrhythmia, and energy metabolism in cardiomyocytes.

6.3. Materials and Methods

6.3.1. General

Two experiments were conducted using 158 and 238 day old commercial male broilers (Ross X Ross 308) randomly allocated to two dietary groups. Each treatment was replicated twice (Exp. 6.1) and thrice (Exp. 6.2) using separate pens, 40 to 50 birds per pen. On day 7, the chickens in respective control and treatment groups were offered either commercial broiler diet containing placebo (condensed extraction medium) or commercial broiler diet mixed with condensed MM extract at a ratio of 4:1 (wt/vol). Hence, the only difference in the diets of two groups was the factors extracted from meat meal. Feed and water were provided *ad libitum*. The experiments were terminated at end of six week of age.

Environmental details, management and feeding regimes were as described previously (Olkowski *et al.,* 1999). Briefly, the birds were housed from day old in environmentally controlled room under constant light. During the first seven days the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 30% (weeks 2, 3) and 40% (weeks 4, 5) lower than that set for normo-thermal brooding. The lowered environmental temperature forces the birds to increase their metabolic rate, which results in increased burden on the cardiovascular system. This approach is very effective in precipitating heart failure practically in all broilers predisposed to heart condition.

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee and the procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

6.3.2. Meat Meal Extraction Procedure and Preparation of Diets

Commercial MM was extracted with methanol acidified with 1% HCl (90:10 v/v) at a ratio 1:4 (wt/vol). First, meat meal was soaked and then stirred with acidified methanol for 40 minutes. The extracted liquid was then filtered and the filtrate was condensed using a rotary evaporator at 45^{o}C.

The treatment and control diets were prepared by thoroughly mixing the condensed MM extract or placebo (condensed extraction medium) with commercial broiler feed. These preparations were spread thin on trays and air dried overnight. The physical characteristic of the feed was not affected by the process of diet preparation. The MM extract added to treatment diet was equivalent to 25% of MM (wt/wt) of the basal diet (i.e the evaporated slurry extracted from 1 kg MM was mixed with 4 kg of feed).

6.3.3. Clinical Monitoring

Birds were monitored several times daily for clinical signs of overt heart disease (fatigue, exercise intolerance, tachypnea, cyanosis, and ascites). The physical examination was performed prior to data collection.

6.3.4. Electrocardiographic Measurements

The ECG measurements were obtained from needle electrodes implanted subcutaneously using a lead II arrangement after induction of light anaesthesia as described previously (Olkowski *et al.,* 1997). The signals from the ECG monitor were digitized using an analog to digital data recording unit and software (Mac Lab and Scope 3.3: AD Instruments Pty Ltd, Castle Hill, Australia) and processed using a

Macintosh computer. Electrocardiographic records were collected from 43 and 31 broilers that appeared normal during routine clinical examination from control and treatment groups respectively during 5^{th} week of age. The ECG data were evaluated for abnormal heart electrophysiological patterns and QRS axis deviation as described previously (Olkowski *et al.*, 1997).

6.3.5. Blood Gas Measurements

Blood gas measurements were obtained from 10 randomly selected birds from each group (5 per pen) at the end of the fifth week of the experiment. For blood gas measurements, approximately 0.5 mL blood samples were obtained anaerobically from the wing vein. The samples were analyzed for pH, pCO_2, pO_2, and hemoglobin O_2 saturation using a pH/Blood Gas Analyzer (Bayer Corporation, East Walpole, MA, USA).

6.3.6. Heart Tissue Procurement for Biochemical Analysis

At the end of the 6^{th} week of the experiment, heart tissue samples for biochemical analysis (high energy phosphates and L-carnitine) were obtained from five randomly selected apparently normal birds from each group and from broilers with clinical signs of chronic heart failure. The hearts were collected immediately following cervical dislocation, snap frozen in liquid nitrogen, and stored as such until analyzed.

6.3.7. Post Mortem Examination

Detailed gross post-mortem examination was performed on all mortalities and birds euthanized during the course of the study. Diagnosis of sudden death syndrome (SDS) was made when death occurred in well grown, apparently normal birds, without any other cause of death evident upon post mortem examination. The diagnosis of congestive heart failure (CHF) was based on findings of gross dilation of the ventricular chambers along with accumulation of ascitic fluid in abdominal cavity. At the termination of experiment all surviving birds were subjected to gross post mortem examination. The diagnosis of sub-clinical heart disease was made when broilers did

not show overt signs of heart failure on routine clinical observation, but post mortem examination revealed gross changes such as atrial and ventricular dilation, and extensive pericardial effusion. The sub-clinical cases were evaluated for presence of cardiac lesions such as dilation of ventricular chambers and the amount of pericardial effusions. The dilations of ventricular chambers (Figure 3.6) were graded as described previously in detail (Olkowski *et al.*, 1998). The pericardial effusions were graded based on the amount of fluid in pericardial sac. Large volume (>5 mL) of pericardial effusion with severely distended pericardium was classified as severe lesion and was considered as a variable of clinical significance (Olkowski *et al.*, 2003b).

6.3.8. Measurements of Cardiac High Energy Phosphates and L-Carnitine

Cardiac creatine phosphate (CrP), adenine triphosphate (ATP), adenine diphosphate (ADP), and adenine monophosphate (AMP) were measured in five randomly selected birds from two pens in each group , and broilers with CHF as previously described (Olkowski *et al.*, 2007a). L-Carnitine was measured as described by Feng *et al.* (2006) with minor modifications. Briefly, samples from the mid portion of left ventricles were homogenized with ice cold phosphate buffer (50 mM, pH 7.4) at a ratio of 200 mg tissue per 1 mL buffer. The homogenates were centrifuged at 2,500×*g* for 10 min at 4°C. The supernatant was precipitated using acetonitrile and methanol (9:1 v/v). A 300 mg mixture of Na_2HPO_4 and Ag_2O (9:1 wt/wt) and 300 mg of KH_2PO_4 were added. This preparation was vortexed for one hour, and following this a derivatizing reagent (40 mg/mL p-bromophenacyl bromide with 50 µL 40% tetrabutylammonium hydroxide) was added into the organic extract. The reaction mixture was incubated at 60°C for 2 hours followed by centrifugation at 12,000×*g* for 15 min, and supernatant was used for analysis. L-carnitine was resolved using a HPLC system (Agilent 1050) with Hyperclone 5 µm CN column (Phenomenex, USA). The mobile phase (90% acetonitrile/10 mM citric-phosphate buffer, adjusted to pH 3) was delivered at a flow rate of 1 mL/min. The elution of carnitine was monitored at 260 nm.

6.3.9. Light and Transmission Electron Microscopy

The heart from three birds fed the control and treatment diets were processed for microscopic examination immediately after cervical dislocation. For light microscopy, samples of heart tissue taken midway from the left ventricular myocardium were fixed in 10% buffered formalin, and following fixation, blocks of myocardium were embedded in paraffin. Longitudinal and transverse sections (5 μm) were processed for light microscopy and stained with hematoxylin/eosin. For electron microscopy samples of heart tissue were fixed in glutaraldehyde and further processed as described previously by Olkowski *et al.* (2001).

6.3.10. Statistical Analysis

Data were analyzed using the microcomputer package Number Cruncher Statistical System (Hintze, 1995). Energy parameters, L-carnitine and blood gas data were analyzed using analysis of variance and means were separated by using Fisher's LSD. The incidence of CHF, SDS and ECG data were analyzed using Fisher's exact test. Morbidity/mortality (CHF and SDS) and descriptive clinical data were analyzed using data combined from experiments 1 and 2. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

6.4. Results

All broilers appeared normal on overt clinical examination at the commencement of the treatment (day 7). The data from both experiments showed consistently that broilers fed diet containing MM extract were at higher risk ($p<0.05$) of succumbing to fulminant CHF than those fed the placebo containing diet (Table 6.1). The incidence of acute heart failure (SDS) was not significantly different ($p>0.05$) between group fed the diet containing MM extract or the diet with placebo.

Table 6.1. Incidence of chronic heart failure (CHF) and sudden death syndrome (SDS) in broilers fed the diet containing MM extract (TRT) and those fed the diet with placebo (CTR).

	Experimental Group	CHF (Mortality/Morbidity)	SDS (Mortality)
Exp. 6.1 (Two replications in each treatment)	CTR (n=83)	38/83 † (45.8 %) ‡	5/83 (6.0 %)
	TRT (n=75)	39/75 (52.0 %)	6/75 (8.0 %)
Exp. 6.2 (Three replications in each treatment)	CTR (n=119)	74/119 (62.2 %)	7/119 (5.9 %)
	TRT (n=119)	88/119 (73.9 %)	8/119 (6.7 %)
Exp. 6.1 & 6.2 Combined Data	CTR (n=202)	112/202 [b] (55.4 %)	12/202 (5.9 %)
	TRT (n=194)	127/194 [a] (65.5 %)	14/194 (7.2 %)
Significance (Fisher's Exact Test)		$p < 0.05$	$p = 0.38$

† Frequency of occurrence; ‡ % of examined; n = Number of birds
Values with different superscripts are significantly different ($P < 0.05$)

Birds with CHF showed severe hypercapnia (pCO_2; 55.0 mmHg), hypoxemia (pO_2; 21.1 mm Hg) and the lowest hemoglobin O_2 saturation (HbO_2Sat; 31.6 %) as compared to birds fed the diet containing MM extract or the diet with placebo (Table 6.2). The blood gas measurements in apparently normal broilers were indicative of hypoxemia in both groups, with values for pO_2 (39.4 mmHg) and HbO_2Sat (72.0 %) in the group fed diet containing MM extract being slightly lower than in broilers fed the diet with placebo [pO_2 (41.5 mmHg) and HbO_2Sat (77.1 %)], but the differences were not significant.

Table 6.2. Comparative study of creatine phosphate (CrP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and L-carnitine content in the left ventricular myocardium in broilers fed the diet containing MM extract (TRT), the diet with placebo (CTR) and broilers with congestive heart failure (Broiler-CHF).

Parameters	CTR	TRT	Broiler-CHF	P Value
pH (Units)	7.42 ± 0.008 ^{b}	7.39 ± 0.021 ^{ab}	7.34 ± 0.019 ^{a}	$p < 0.001$
pCO_2 (mm Hg)	45.1 ± 1.32 ^{a}	45.6 ± 1.42 ^{a}	55.0 ± 4.13 ^{b}	$p < 0.01$
pO_2 (mm Hg)	41.5 ± 1.54 ^{b}	39.4 ± 2.02 ^{b}	21.1 ± 2.12 ^{a}	$p < 0.01$
Hb O_2 Sat %	77.1 ± 1.76 ^{b}	72.0 ± 3.65 ^{b}	31.6 ± 6.07 ^{a}	$p < 0.01$
CrP	1.06 ± 0.020 ^{b}	0.81 ± 0.025 ^{a}	0.76 ± 0.014 ^{a}	$p < 0.001$
ATP †	1.52 ± 0.045 ^{b}	1.35 ± 0.108 ^{b}	1.06 ± 0.096 ^{a}	$p < 0.01$
ADP †	0.86 ± 0.017 ^{b}	0.66 ± 0.052 ^{b}	0.731 ± 0.027 ^{ab}	$p < 0.05$
AMP †	0.28 ± 0.035	0.33 ± 0.034	0.37 ± 0.052	$p = 0.38$
L-Carnitine †	0.30 ± 0.033	0.24 ± 0.070	0.16 ± 0.013	$p = 0.16$

† Units: CrP, ATP, ADP and AMP (μg/mg of heart tissue); L-Carnitine (μM)/mg heart tissue). Values are means ± SE, Means within rows with different superscripts are significantly different ($P< 0.05$)

Post mortem examination in all birds showing signs of CHF revealed gross dilation of the ventricles and severe pericardial effusions along with ascitic fluid in abdominal cavity. There were no differences in gross features of heart pathology between CHF broilers from the placebo group and the CHF broilers fed diets spiked with MM extract. However, among apparently normal birds from both groups that showed pathological signs of sub-clinical heart disease, the occurrence of severe ventricular dilation was higher ($p<0.05$) in the group fed the diet containing MM extract (59.5% of examined i.e. 44 out of 74), as compared to the placebo group (40.7% of examined i.e. 22 out of 54). Proportion of broilers with severe pericardial effusions (>5 ml) tended to be higher ($p=0.06$) in the group fed the diet containing MM extract (44.6% of examined i.e. 33 out of 74) as compared to broilers on placebo group (27.8% of examined i.e. 15 out of 54).

Electrocardiographic evaluation revealed that 3 out of 43 (6.97%) broilers fed the control diet, and 4 out of 31 (12.9%) broilers fed the treated diet showed arrhythmia. Cardiac rhythm abnormalities included atrio-ventricular blocks, and episodes of atrial and ventricular arrhythmia, but most commonly premature ventricular contractions (PVC) were observed. Analysis of QRS axis deviation revealed that 19 out of 43 (44.2%) broilers fed the placebo diet, and 16 out of 31 (51.6%) broilers fed diet spiked with MM extract showed left axis deviation, an early sign of CHF. The heart rate in apparently normal broilers from group fed placebo containing diet (308 ± 6.4 beats/minute) was similar to group fed MM spiked diet (310 ± 7.0 beats/minute).

Histo-pathological examination revealed degenerative changes in the myocardium of apparently normal broilers from both groups, but there were considerable difference in the magnitude of the lesions (Figure. 6.1).

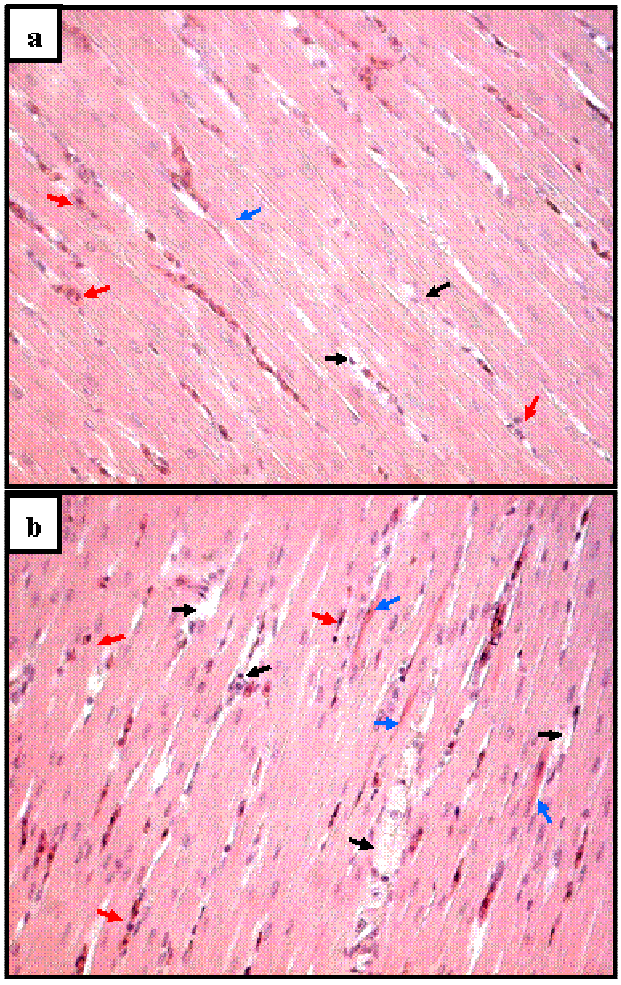

Figure 6.1. Representative histo-pathological features of the mural left ventricular myocardium in a broiler from the group fed the placebo diet (a) and broilers fed the diet containing meat meal extract (b). Original magnification 400X.
In broilers with sub-clinical heart condition, typical lesions in the ventricular myocardium consisted of degenerative changes in the cardiomyocytes characterized by distinct dull dark pink appearance of affected cardiomyocytes with cytoplasmic eosinophilia (blue arrows) and showed evidence of nuclear pyknosis and karyorrhexis (red arrows). Some cardiomyocytes showed vacuolated cytoplasm and degenerative changes in nuclei indicative of advanced changes (black arrows). Notably, the lesions appear to have similar qualitative features in both groups, but in comparison to birds from the placebo group (a), those fed the diet containing MM extract (b) showed more extensive degenerative changes in the ventricular myocardium.

Ultrastructural examination revealed morphological changes in mitochondrial architecture, characterized by swelling, vacuolization and destruction of matrices and cristae (Figure 6.2). Overall, cardiomyocytes from broilers fed the placebo containing diet showed mostly normal morphology of mitochondria, but mild to moderate changes were apparent in some clusters of mitochondria (Figure 6.2.a). In contrast, cardiomyocytes of broilers fed the diet containing MM extract contained numerous mitochondria showing more advanced morphological changes (Figure 6.2.b).

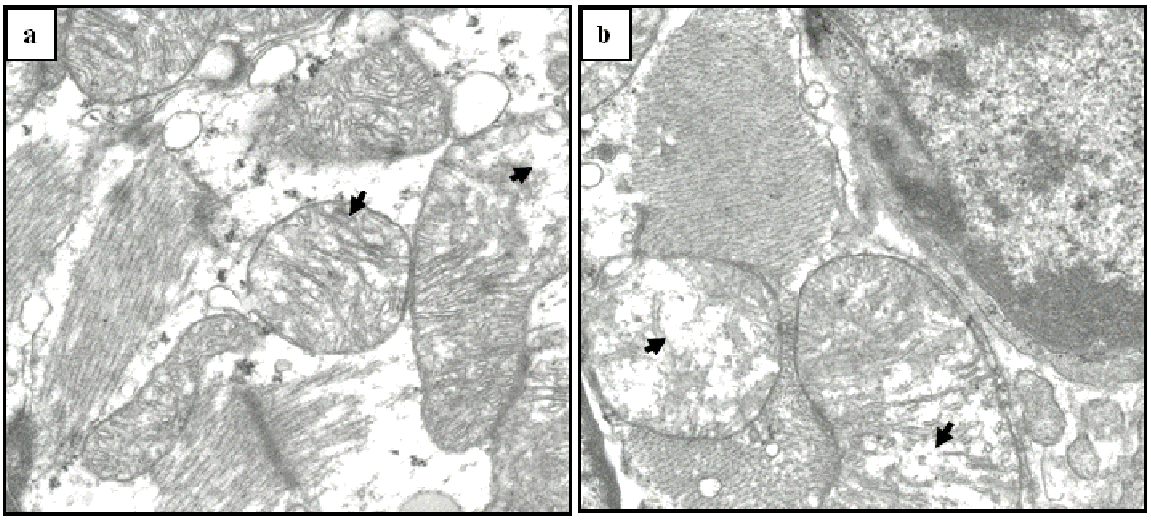

Figure 6.2. Representative transmission electron micrographs from left myocardium of a broiler fed placebo containing diet (a) and a broiler fed diet containing MM extract (b).
For the most part, myocardial mitochondria showed normal morphology with well defined and dense cristae, but in some areas of the cardiomyocytes degenerative changes in the mitochondrial structure were evident. Examples of typical changes in mitochondrial morphology in broilers fed placebo containing diet (a) and broilers fed diet spiked with MM extract (b). It is noteworthy that the changes in the mitochondria (arrows) such as swelling, vacuolization, and destruction of matrices and cristae were qualitatively evident in both groups, but are more severe in broilers fed diet spiked with MM extract in comparison to broilers fed placebo containing diet.

Cardiac levels of ATP and CrP were significantly ($p<0.01$) lower in broilers with CHF (Table 6. 2). The levels of cardiac ATP and CrP were approximately 16% and 24% lower in birds fed diet spiked with MM extract as compared to birds fed placebo diet, but this difference was statistically significant ($p<0.05$) only for CrP. The ADP level was lower ($p<0.05$) in birds fed the diet spiked with MM extract, as compared to birds fed the placebo diet. Cardiac AMP level was higher by 17.1% and L-carnitine level was lower by 18.9% in birds fed the diet containing MM extract than in those fed the placebo diet.

6.5. Discussion

The findings from the present study indicate that methanol extracted factors from meat meal significantly increase the risk of CHF in fast-growing broilers. The nature and severity of changes in the heart muscle seen on gross examination, as well as on light microscopy and electron microscopy levels, were consistently more pronounced in birds fed diet containing MM extract as compared to birds fed the placebo diet. Taken together, these findings indicate that MM used in broiler diets may contain appreciable quantities of compounds capable of inducing cardiac muscle lesions and precipitate heart failure.

The present study showed that adverse effects observed in the hearts of broilers fed diets spiked with MM extracts are indeed associated with putative cardiotoxic compounds present in MM. Although, the identification of specific components of our extract was beyond the scope of present study, it is reasonable to assume that the adverse cardiac effects observed in the present study were associated with several putative cardiotoxic compounds present in MM and those generated during thermal processing of MM were likely of major significance.

Among many compounds that may be produced during high temperature meat processing, the most recognized are heterocyclic amines (HA). Synthesis of HA during thermal treatment of proteins is well documented in the literature (Davis *et al.,* 1994; Gaubatz, 1997; Dubuisson *et al.,* 2001; Bordas *et al.,* 2004). Thus far, twenty HA

generated in cooked meat have been identified (Bordas *et al.,* 2004). The amounts and types of HA formed during cooking can be attributed to parameters such as time and temperature (Gross *et al.,* 1993; Knize *et al.,* 1998), and in these terms, the processing technology used in rendering process of animal byproducts or thermal treatment of plant proteins provide ideal conditions for generation of cardiotoxic HA. Therefore, given the fact that production of MM involves long term, high temperature treatment during the rendering process, it is reasonable to assume that these compounds are present in MM used for broiler diets.

The histo-pathological changes in the cardiomyocytes of broilers exposed to MM extract are consistent with lesions associated with cardiotoxic effects of HA in experimental animals, such as myocyte necrosis and degeneration, myofibrillar loss, swelling and vacuolization of mitochondria described by Davis *et al.* (1994). In particular, morphological changes in the mitochondria seen in our broilers are remarkably similar to the lesions observed in the mitochondria of monkeys treated with purified 2-amino-3-methylimidazo quinoline (Thorgeirsson *et al.,* 1994), and in rats treated with 2-amino-1-methyl-6-phenylimidazo pyridine (Takahashi *et al.,* 1996). Of note, these compounds are major HA produced during thermal processing of meat (Gross *et al.,* 1993; Guy *et al.,* 2000; Turesky *et al.,* 2005).

The present study provides evidence linking the morphological changes in the mitochondria with biochemical dysfunction in cardiac energy metabolism. Interestingly, closer scrutiny of the biochemical data from apparently normal broilers fed the placebo diet, those fed diets spiked with MM extract, and broilers with CHF revealed an apparent trend that deserves further consideration. In order to understand the trends in energy parameters in these three groups of birds, the measurements from birds fed the diet containing MM extract and birds with CHF were expressed with reference to levels in broilers fed the placebo diet taken as 100%. These comparisons are presented in Figure 6.3.

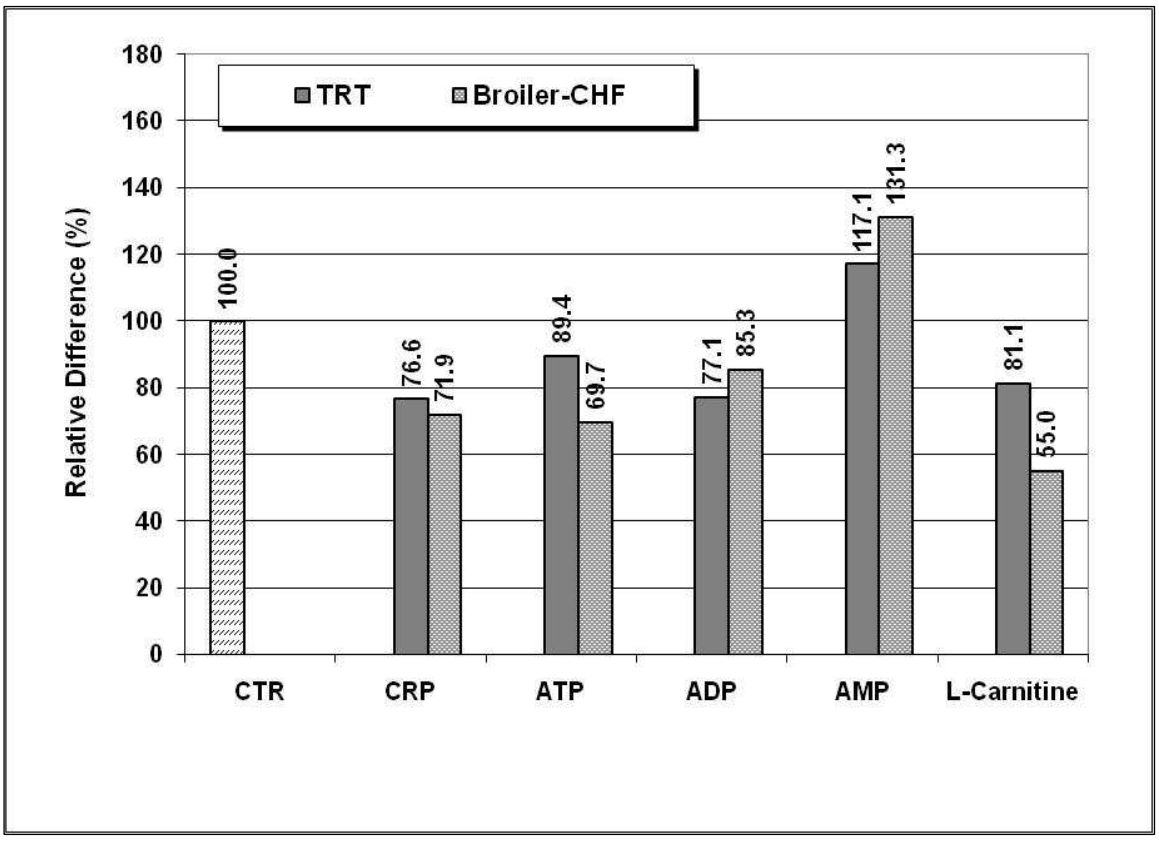

Figure 6.3. Relative changes in cardiac creatine phosphate (CrP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and L-carnitine content in broilers fed diet spiked with MM extract (TRT) and in broilers with congestive heart failure (Broiler-CHF) expressed on a percentage basis of data from broilers fed placebo diet (CTR) representing on the graph a reference value of 100.

Notably, the lower status of the parameters critical in cardiac energy metabolism such as CrP, ATP in broilers fed the diet spiked with MM extract relative to the broilers fed the placebo diet indicate that this apparent biochemical defect in energy substrate metabolism can be directly correlated with the extent of morphological changes seen in the mitochondria.

Recently insufficiency of energy substrate has been linked with the deterioration of heart function in broilers (Olkowski *et al.*, 2007a). The loss of contractile function of the heart is associated with mitochondrial inability to supply ATP and to replenish CrP

(energy reservoir) from creatine in the myocardium, leading to a state of energy deprivation in the heart (Stanley *et al.*, 2005).

The present study provides proof of concept that MM used in commercial broiler diets may contain compounds capable of inducing morphological and biochemical changes in the heart tissue. In feed industry, MM and FM are used as crude protein supplements in poultry diets. However, it is a well known fact that the quality of these products (particularly MM) is highly variable. Undoubtedly, depending on animal by-products used, and the different rendering technologies, there is possibility that some MM may contain high levels of cardiotoxic compounds. In the context of our findings, further studies are needed to evaluate the risks associated with various MM and FM products, establish safe maximum levels of inclusion, and develop rendering processing guidelines and quality control.

7. THE ROLE OF OXIDATIVE STRESS IN THE DEVELOPMENT OF CONGESTIVE HEART FAILURE IN A CHICKEN GENOTYPE SELECTED FOR RAPID GROWTH

7.1. Abstract

The present study examined the possible role of reactive oxygen species in the pathogenesis of heart failure in broilers. Data were collected from four groups of birds at various risk of heart failure i.e. leghorn chickens (resistant to heart failure), slow-growing feed restricted broilers (low risk of heart failure), fast-growing *ad libitum* fed broilers (high risk of heart failure), and broilers with congestive heart failure (CHF). In the first part of the study, basic clinical parameters and ultrastructural changes were examined in the context of lipid peroxidation of the ventricular myocardium. This was followed by the study of *in vitro* changes in the activity of selected cytosolic enzymes (creatine kinase: CK, and lactate dehydrogenase: LDH) and mitochondrial enzymes (pyruvate dehydrogenase: PDH and α-ketoglutarate dehydrogenase: α-KGDH) in the presence of oxidants (H_2O_2 or tertiary butyl hydroperoxide). The distinctive clinical feature in the fast-growing broilers and in the broilers with CHF as compared to slow-growing broilers or leghorn chickens was a significant decline in heart rate ($p<0.05$). Electron microscopy revealed marked morphological changes in myocardial mitochondria in these broilers i.e. fast-growing broilers and broilers with CHF. The level of malondialdehyde equivalents, an indicator of lipid peroxidation subsequent to generated oxidative stress, was significantly higher ($p<0.05$) in *ad libitum* fed broilers and highest ($p<0.01$) in broilers with CHF. *In vitro*, the presence of oxidants had a detrimental effect on CK and α-KGDH activity, while LDH activity increased. The activity of PDH was not altered by oxidants. Findings from the present study indicate that the deterioration of heart function in fast-growing commercial broilers is associated with oxidative stress leading to lipid peroxidation of cellular and mitochondrial

membranes, and decreased activity of myocardial CK and α-KGDH enzymes critical for energy synthesis and transformation pathways.

7.2. Introduction

The modern fast-growing broilers are inherently predisposed to heart conditions. This predisposition developed due to continuous selection for traits of economic importance (fast growth and feed conversion efficiency) without due consideration of health related parameters (Flock *et al.,* 2005). One of these health related problems is congestive heart failure (CHF) observed clinically in many fast-growing commercial broilers. The impact of this disorder has been estimated around 1 billion dollars annually (Maxwell and Robertson, 1997). Previous studies from our lab revealed that a relatively large number of modern commercial broilers reared under subnormal temperature, show evidence of hypoxemia associated with sub-clinical heart disease (Olkowski *et al.,* 2005b; Nain *et al.,* 2008b). Oxidative metabolism is a normal process in all tissues. Cardiomyocytes require a constant supply of O_2 for normal cardiac functions. However, oxygen associated metabolism in myocardium sometimes can contribute to cardiac dysfunction, and may ultimately lead to heart failure (Giordano, 2005; Redout *et al.,* 2007).

During the normal oxidative metabolic process various reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. During this normal metabolism 1-2% of oxygen is converted to ROS (Sheeran and Pepe, 2005). Several specialized metabolic mechanisms normally dispose off these very harmful reactive species. However, under some circumstances, increased ROS/RNS production or decreased antioxidant defenses may lead to oxidative stress where the generated reactive species can alter the properties of lipids, proteins, and nucleic acids, leading to cellular dysfunctions (for review see Orrenius *et al.,* 2007). Recent research findings from different laboratories suggest that ROS and RNS play a critical role in development of human heart failure (Andreka *et al.,* 2004; Sam *et al.,* 2005; Nediani *et al.,* 2007).

Lipid peroxidation can alter membrane properties of cellular and subcellular organelles (mitochondria and sarco-endoplasmic reticulum) crucial for maintenance of normal cardiomyocyte function. Broilers with CHF show evidence of calcium overload in these sub-cellular components (Maxwell *et al.*, 1993; Li *et al.*, 2006), and evidence of breakdown and release of the protein of contractile apparatus, such as myosin and troponin T, into the circulation (Maxwell *et al.*, 1994).

The role of oxidative stress has long been debated in the pathogenesis of heart failure in human and animal models of cardiomyopathy. However, limited research has been done to investigate the possible involvement of oxidative stress in CHF in broilers. Recent studies from our laboratory revealed that energy metabolism is compromised in broilers developing heart failure (Olkowski *et al.*, 2007b; Nain *et al.*, 2008b). In order to further understand the physiological and biochemical disturbances leading to congestive heart failure in fast-growing commercial broilers, we were interested to examine the possible role and molecular mechanisms of oxidative stress in the pathogenesis of chronic heart failure. In order to explain the molecular mechanisms underlying the pathogenesis of heart failure in commercial broilers we compared clinical, morphological, molecular and biochemical parameters among chickens that were categorized according to risk of developing heart failure as follows: 1) leghorn chickens (resistant to heart failure); 2) feed restricted, slow-growing broilers (low risk of heart failure); 3) *ad libitum* fed, fast-growing broilers (high risk and incidence of heart failure) and 4) broilers showing signs of congestive heart failure (broilers with CHF, observed within *ad libitum* fed group). Firstly, oxidative stress associated with the deterioration of heart function was measured. Secondly, we examined the impact of simulated oxidative stress on the activities of essential enzymes involved in cardiac energy synthesis and transformation pathways.

7.3. Materials and Methods

7.3.1. Animals and Management

The birds (all males) used to measure specific parameters represented a random sample obtained from basic flocks consisting of 48 leghorns, 39 feed-restricted slow-growing broilers, and 83 fast-growing *ad libitum* fed broilers. The birds were allocated to four pens, approximately 40 to 50 birds per pen. The leghorns and feed-restricted and ad libitum fed broilers were offered with standard commercial broiler diet. The group of broilers designated as slow-growing was subjected to a feed restriction regime (70% *ad libitum* fed group fed once a day) from day 7 onwards. All other groups of birds were fed *ad libitum.* During the first 7 days the brooding temperature was maintained at 34°C followed by a gradual decrease to a level approximately 30% (weeks 2, 3) and 40% (weeks 4, 5) lower than that set for normo-thermal brooding. We have extensively tested and validated this management protocol and we routinely use it in our laboratory (Olkowski *et al.*, 1999, 2005b, 2007a; Nain *et al.*, 2008b). Lowered thermal brooding temperature forces birds to increase their metabolic rate, which results in increased burden on the cardiovascular system and subsequent precipitation of heart failure in broilers predisposed to heart related conditions. Relative to the *ad libitum* fed group, feed restriction decreases body weight gain of broilers by 25 to 30%, and this slower growth rate reduces the risk of heart failure practically to zero.

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee. The procedures were performed as per the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

7.3.2. Clinical Evaluation

Birds were monitored daily for overt signs of heart disease such as fatigue, exercise intolerance, tachypnea, hypoxemia, generalized cyanosis, and ascites. Birds *in extremis* observed with in *ad libitum* fed group were euthanized. Detailed physical examination was performed prior to data collection. Heart rate measurements were

obtained using electrocardiography (ECG) as described previously (Olkowski and Classen, 1998a) from 10 birds from each group at the end of the fifth week of the experiment. Birds showing above listed signs were classified as broilers with CHF.

7.3.3. Transmission Electron Microscopy (TEM)

To determine the ultrastructural changes in myocardium of birds at various risk of heart failure, hearts obtained from leghorn chickens, fast-growing *ad libitum* fed broilers, and broilers with CHF were processed for TEM. Heart tissue samples from the mid portion of the left ventricular wall were processed as described previously (Olkowski *et al.*, 2001).

7.3.4. Heart Tissue Procurement for Biochemical Analysis

Heart tissue samples used for biochemical analysis were obtained from five randomly selected birds from each group as described above, at the end of the 6^{th} week of the experiment. Hearts were collected following cervical dislocation and snap frozen in liquid nitrogen and were stored in liquid nitrogen until analyzed.

7.3.5. TBARS Assay

Thiobarbituric acid reacting substances (TBARS), an indicator of oxidative damage were measured spectrophotometrically as described previously by Ohkawa *et al.* (1979) with modifications. Briefly, frozen/pulverized tissue samples from mid portion of left ventricular myocardium were homogenized first in KCl mixture (2mM EGTA, 0.02% BHT in 1.15% KCl) using microtube homogenizer and then add RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 1% Sodium deoxycholate and 1% SDS, with pH adjusted to 7.2 using NAOH) and then mix. Finally, 1 g of heart tissue was present in 6 ml of solution containing RIPA buffer and KCl mixture in a ratio of 4:5 v/v. The homogenate was centrifuged at 15000×*g* for 10 min at 4°C. 200 µL of supernatant was mixed with 0.2 mL 8.1% SDS, 2500 µL 30% acetic acid (pH adjusted to 3.5), 375 µL 0.8% TBA and 8.25 µL of 0.02% BHT aqueous solution followed by incubation at 95°C for 1h. After incubation and subsequent

cooling, an equal volume of n-butanol/pyridine mixture (15:1 v/v ratio) was added. After vigorous shaking, it was centrifuged at 4000×*g* for 10 min. The organic layer obtained at the top following centrifugation was used for measuring thiobarbituric reactive substances at 532nm. A calibration curve was prepared using a malondialdehyde (MDA) standard, and the dilution was made in such a way that the expected concentration of each sample falls in the middle range of the calibration curve. The coefficient of determination approached 0.99 for the standard curve. All samples were analyzed in duplicate in the same assay to avoid interassay variability.

7.3.6. *In Vitro* Enzyme Inhibition

Activity of selected cytosolic (Creatine Kinase: CK and Lactate Dehydrogenase: LDH) and mitochondrial (Pyruvate Dehydrogenase: PDH and α-Ketoglutarate Dehydrogenase: α-KGDH) enzymes were tested using an *in vitro* indicator of oxidative stress i.e. H_2O_2 or tertiary butyl hydroperoxide (TBH) or both. Enzyme sensitivity measurements were performed in heart tissue obtained from the mid portion of the left ventricle free wall of fast-growing *ad libitum* fed broilers.

The activities of CK, LDH, PDH and α-KGDH were measured as described previously (Nain *et al.*, 2008b). Briefly, aliquots of 300 mg of frozen samples were homogenized in phosphate buffer (50 mM, pH 7.4) in the ratio of 1:10 (100 mg tissue : 1 mL buffer) in test tubes pre-cooled with ice. The homogenate was centrifuged at 2,500×*g* for 10 min at 4°C and the suspension was further centrifuged at 12,000×*g* for 10 min. The supernatant was used for CK and LDH measurements. The CK activity was measured using a CK kit (Roche Diagnostics, Indianapolis, IN, USA) while LDH activity was measured using LD-L10 (Sigma Diagnostics Inc., St. Louis, MO, USA). The mitochondria containing pellet was further washed three times with phosphate buffer to remove remnants of cytosolic enzymes and finally this pellet was resuspended in Tris buffer (50 mM, pH 7.6 with 0.5% Triton). PDH and α-KGDH activity were assayed in mitochondrial extracts. PDH activity was measured using a cocktail, where final components of the incubation media were 2 mM pyruvate, 2.5 mM NAD, 0.15 mM flavin adenine dinucleotide, 2 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.13

mM Coenzyme A, 2.6 mM dithiothreitol, and 30 mM Tris buffer at pH 7.2. The α-KGDH activity was measured using a cocktail containing 3.2 mM α-keto glutaric acid, 2 mM NAD, 0.5mM Coenzyme A, 0.7 mM thiamine pyrophosphate, and 1 mM $MgCl_2$. The assays were validated for the linearity of responses for time of reaction and protein content.

The inhibition study of all the above mentioned enzymes was performed in presence of various concentrations of H_2O_2 (see Figure 7.2). In addition, the activities of PDH and α-KGDH were also assessed in the presence of serial dilutions of TBH (Figure 7.2). Prior to final measurements each component of the cocktail mixture for every enzyme was incubated in the presence of H_2O_2 or TBH, to establish that the results obtained are purely subsequent to enzyme oxidation.

The reaction was initiated by adding 20 µL of cytosolic or mitochondrial fraction in 200 µL of cocktail plus 5 µL of serially diluted H_2O_2 or TBH per well in a 96 well micro plate pre-incubated at 37°C for all of the above mentioned enzymes. Enzyme activity measurements were performed at 340 nm using a microplate reader SpectraMax Plus (Molecular Devices, CA, USA). The final measurements were performed during the linear phase of responses pre-established during validation phase as described previously. The inhibition constant (IC_{50}) was used to know the concentration of the oxidants required for 50% inhibition of an enzyme activity. IC_{50} values were calculated by non linear regression analysis using a GraphPad Prism 3.03 (GraphPad Software, San Diego, CA).

7.3.7. Statistical Analysis

Statistical analyses were carried out by GLM ANOVA from the microcomputer package Number Cruncher Statistical System (Hintze, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

7.4. Results

Overall, fulminant CHF (based on mortality and morbidity data) was observed in 38 out of 83 (46%) broilers from the *ad-libitum* fed group. None of the birds from the feed restricted and leghorn group showed clinical signs indicative of heart disease.

The cardiac measurements revealed a marked ($p<0.05$) decline in heart rate (HR: 308 beats/min) in the *ad libitum* fed group as compared to the feed restricted (HR: 371 beats/min) and leghorn chickens (HR: 372 beats/min) (Table 7.1). Profound bradycardia (HR: 236 beats/min) was observed in broilers with fulminant CHF.

Table 7.1. Heart rate and malondialdehyde (MDA) equivalent levels (nmol/mg of heart tissue) measured from leghorn chickens, feed restricted slow-growing broilers (Broiler-Res), fast-growing broilers fed *ad libitum* (Broiler-AL), and in broilers with congestive heart failure and ascites (Broiler-CHF).

Measurements	Leghorn	Broiler-Res	Broiler-AL	Broiler-CHF
Heart Rate (beats/min)	372 ± 5.7^{c}	371 ± 3.6^{c}	308 ± 6.4^{b}	236 ± 8.2^{a}
MDA Equivalents (nmol/mg of heart tissue)	0.91 ± 0.045^{a}	0.99 ± 0.038^{a}	1.19 ± 0.087^{b}	1.41 ± 0.025^{c}

Values are means ± SE. Means within rows with different superscripts are significantly different ($p< 0.05$).

Post mortem examination was performed on birds that were euthanized or died during the course of study. Broilers with CHF showed gross dilation of the ventricular chambers along with pulmonary congestion. Approximately 80 to 90% of broilers showing these changes also showed severe pericardial effusion and the accumulation of ascitic fluid in the abdominal cavity.

Ultrastructural examination revealed significant morphological changes in the cardiomyocyte contractile apparatus and mitochondria. In some areas disintegration or even total dissolution of myofibrillar proteins was observed. Mitochondrial lesions were seen in some apparently normal broilers, but predominantly in broilers with CHF.

Major morphological changes in the mitochondria include pleomorphism, swelling and vacuolization along with disintegration and loss of cristae.

A distinctive feature in fast-growing *ad libitum* fed broilers, as compared to leghorn chickens, was that the inter-fibrillar mitochondria were numerous and appeared in large clusters. Careful evaluation of electron micrographs (Figure 7.1) revealed that changes were most pronounced in broilers with CHF.

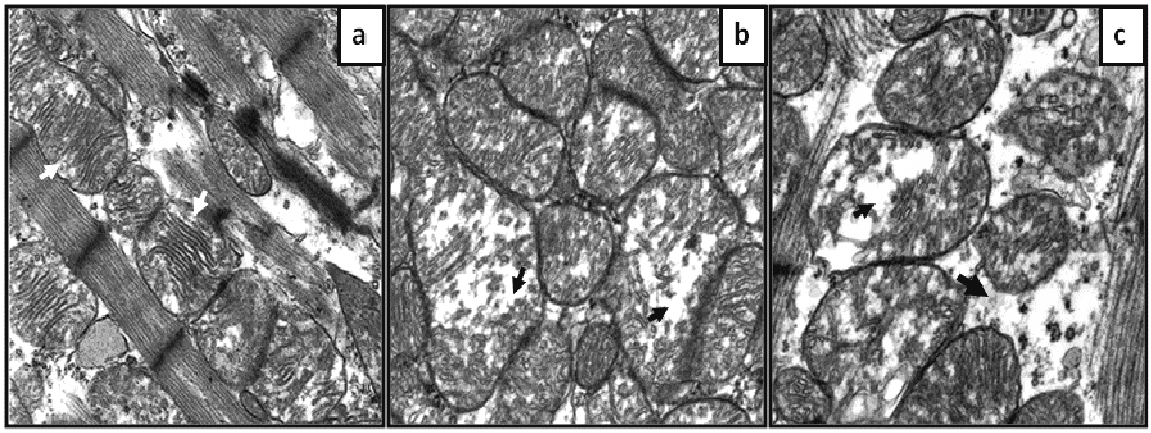

Figure 7.1. Representative transmission electron micrographs from left ventricular myocardium of a leghorn chicken (resistant to heart failure) (a), fast-growing ad libitum fed broiler (high risk of heart failure) (b) and from a broiler that developed CHF (c). Original magnification 10,000X.
Comparable presentation of electron micrographs from various categories of birds revealing various degrees of mitochondrial lesions. **a)** Micrographs from leghorn revealing mitochondria are monomorphic with well defined cristae (white arrow) and mitochondrial membrane is intact. **b)** Micrographs from fast-growing *ad libitum* fed broiler showed various degrees of changes to mitochondrial architecture, some mitochondria are normal while few revealed vacuolization and disintegration of cristae (black arrow). **c)** Micrographs from broiler with CHF, note mitochondrial swelling, vacuolization and disintegration of cristae (black arrow).

Findings from the TBARS assay showed that lipid peroxidation was highest ($p<0.05$) in broilers with CHF as compared to slow-growing feed restricted broilers, fast-growing *ad libitum* fed broilers and leghorn chickens (see Table 7.1). The findings from this assay further revealed that fast-growing *ad libitum* fed broilers had higher ($p<0.05$) lipid peroxidation as compared to slow-growing feed restricted broilers and

leghorn chickens. The values from slow-growing feed restricted broilers and leghorn chickens did not differ significantly ($p>0.05$).

In the first part of the study it was ensured that kit/cocktail components (CK and LDH kit, α-KGDH and PDH cocktail components) were not affected by the presence of oxidants (Table 7.2). *In vitro* enzyme inhibition studies revealed decreased CK activity (IC_{50} = 5.71 mM) in the presence of increasing H_2O_2 concentrations (Figure 7.2). However, LDH activity was increased in the presence of H_2O_2 at a concentration above 12.2 mM. The PDH activity was not affected by the presence of H_2O_2 and tertiary butyl hydroperoxide. α-KGDH activity was not affected by the presence of H_2O_2 but decreased in the presence of increasing concentration of tertiary butyl hydroperoxide (IC_{50} = 6.25 mM).

Table 7.2. Effect of presence of oxidants (H_2O_2 or tertiary butyl hydroperoxide (TBH)) on enzyme kit/cocktail components (CK and LDH kit, PDH and α-KGDH cocktail) and on adenosine triphosphate (ATP) and nicotine adenine dinucleotides (NAD and NADH) components on absorbance.

	Enzyme activity (OD@340nm)			
Reaction Components	CK	LDH	PDH	α-KGDH
Kit/cocktail + H_2O_2/TBH	ND*	ND	ND	ND
Kit/cocktail + Enzyme	100%	100%	100%	100%
Kit/cocktail + Enzyme + H_2O_2/TBH	INH**	ACT***	NO****	INH/NO*****
ATP + H_2O_2/TBH	ND	NA	NA	NA
NAD + H_2O_2/TBH	ND	ND	ND	ND
NADH + H_2O_2/TBH	ND	ND	ND	ND

* ND: No detectable change; ** INH: Activity inhibition observed at various concentration of H_2O_2;***ACT: Increased activity in presence of H_2O_2; ****NO: No change in activity in presence of H_2O_2/TBH; ***** INH/NO: Activity inhibition observed in presence of TBH/No change in activity in presence of H_2O_2; NA: Not applicable

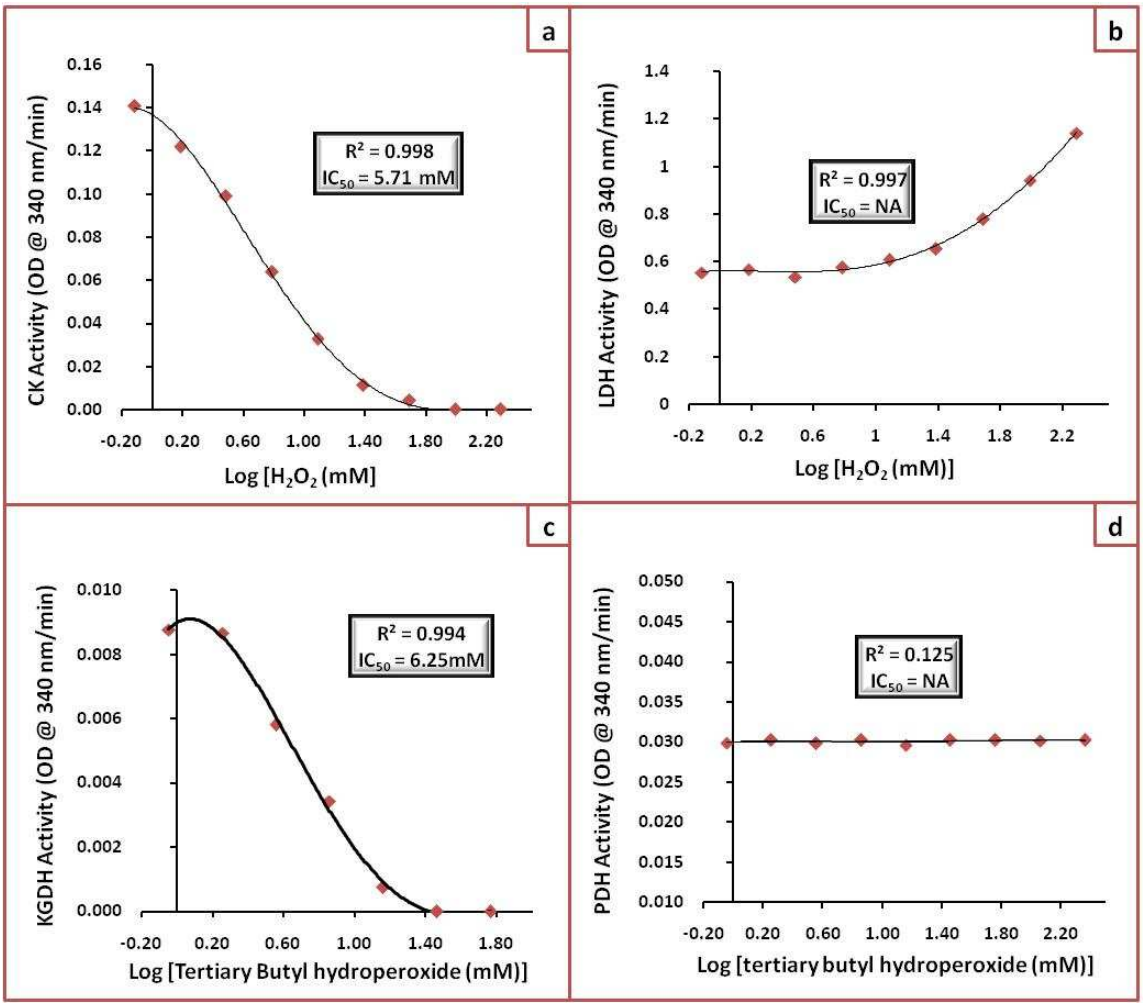

Figure 7.2. The creatine kinase (CK), pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH) and alpha-ketoglutarate dehydrogenase (α-KGDH) enzyme sensitivity to oxidative stress tested in presence of H_2O_2 or tertiary butyl hydroperoxide (TBH).
a) CK activity (optical density; OD measured at 340nm), note decreased activity (IC_{50} = 5.71 mM) as concentration of H_2O_2 increases. b) LDH activity (OD measured at 340nm), note activity of LDH increases with increased concentration of H_2O_2. c) α-KGDH activity, note decreased activity (IC_{50} = 6.25 mM) as concentration of tertiary butyl hydroperoxide increases. d) PDH activity, no affect on activity in the presence of tertiary butyl hydroperoxide.

7.5. Discussion

Several findings from the present study provide evidence that deterioration in heart function in broilers is associated with oxidative stress. The morphological changes observed in cardiac mitochondria in *ad libitum* fed broilers and in broilers with CHF are consistent with damage associated with oxidative stress, and this is well supported by biochemical evidence of damage to mitochondrial membranes caused by lipid peroxidation.

The leghorn chickens in the present study did not show any sign of heart failure; hence measurements from the leghorns were used as reference values. In order to understand the trends in the measured parameters (MDA equivalents and heart rate), the values from the slow-growing feed restricted broilers (low risk of heart failure), fast-growing *ad libitum* fed broilers (high risk of heart failure) and broilers with CHF (observed within ad libitum fed group) were plotted with respect to levels observed in leghorns (resistant to heart failure) taken as 100% (Figure 7.3).

The present findings further confirmed that fast-growing commercial broilers are at increased risk of heart failure. This increased risk of heart failure in broilers was associated with deterioration of heart functions as revealed by lowered heart rate observed in *ad libitum* fed broilers and in broilers with CHF as compared to feed restricted slow-growing broilers and leghorn chickens.

Declining heart rate is the most characteristic early patho-physiological feature of deterioration of heart pump function in fast-growing broilers. This has been reported in previous studies from our laboratory (Olkowski and Classen, 1998b; Olkowski *et al.,* 2005a; 2007a, Nain *et al.,* 2008b), as well as studies of others (Deng *et al.,* 2006; Druyan *et al.,* 2007), and has been confirmed in the present study. Interestingly, as demonstrated in Figure 7.3, the pattern of lowered heart rate appears to be strongly correlated with increasing levels of TBARS. This indicates that the deterioration of heart function is associated with oxidative damage in the myocardium.

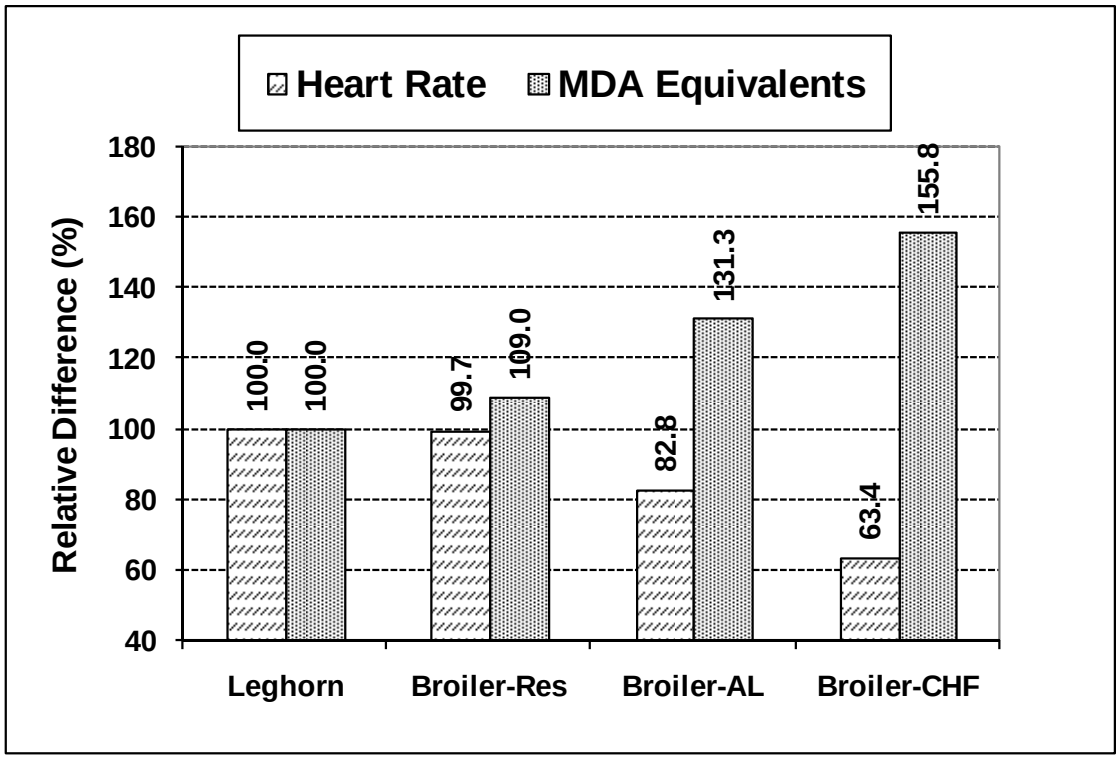

Figure 7.3. Relative heart rate and level of malondialdehyde (MDA) equivalents in feed restricted slow-growing broilers (Broiler-Res, low risk of heart failure), *ad libitum* fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with congestive heart failure (Broiler-CHF) expressed on percentage basis of leghorn chickens (Leghorn, resistant to heart failure).

Morphological changes observed in myocardial mitochondria in the present study are consistent with oxidative damage. Notably, mitochondria are the major source of ROS, but because of their very high component of membranes, they are also a very sensitive target of reactive oxygen species attack. The membrane lipids are very sensitive to oxidative damage due to the presence of polyunsaturated fatty acids, subsequently leading to lipid peroxidation (Halliwell and Gutteridge, 1985). Currently, one of the most common and well recognized approaches to measure the effects of free radicals is by measuring the oxidative damage (i.e. lipid peroxidation) to cellular membranes (Lykkesfeldt and Svendsen, 2007).

The measurements from the lipid peroxidation in the present study showed that in fast-growing broilers oxidative stress increases as risk of heart failure increases (Figure 7.3). Moreover, the observed oxidative damage can readily be associated with morphological changes in the mitochondria.

The biochemical evidence of oxidative damage (elevated TBARS) corresponds well with the observed morphological changes in the mitochondria such as mitochondrial swelling, vacuolization, loss and disintegration of cristae. Earlier studies from our lab have reported insufficiency of high energy phosphates in the myocardium of broilers developing CHF (Olkowski *et al.,* 2007a; Nain *et al.,* 2008b). Hence, we can argue that mitochondrial damage corresponds with the lowered energy reserve in myocardium as the mitochondrion is the power house of the myocardial cell.

Heart is one of the greatest energy consuming organs in the body, which requires a constant supply of O_2 to maintain its metabolic functions (Giordano, 2005). In heart mitochondria comprise 30% of the cardiomyocyte volume (Sheeran and Pepe, 2006). The major sites of ROS formation are at complex I and complex III of electron transport chain located in inner membrane of mitochondria (Turrens and Boveris, 1980; Turrens *et al.,* 1985). During normal metabolism 1-2% of oxygen is converted to ROS. Increased ROS or RNS production or decreased antioxidant defenses leads to oxidative stress.

α-KGDH, one of the key rate limiting enzymes of the TCA cycle is involved in energy synthesis pathways. α-KGDH acts as a source as well as sensitive target of H_2O_2 in the mitochondria (for review see Tretter and Adam-Vizi, 2005). Studies in rats have demonstrated that α-KGDH is a sensitive target of H_2O_2 (Nulton-Persson and Szweda, 2001). On the other hand, recent studies have demonstrated that this enzyme itself is able to generate ROS, leading to a state of oxidative stress (Starkov *et al.,* 2004; Tretter and Adam-Vizi, 2004). Although the activity α-KGDH does not appear to be directly sensitive to H_2O_2, it is notable that this enzyme was sensitive to TBH. In this

context it is important to stress that TBH can produce both alkoxyl and hydroxyl radicals, two of the most potent ROS.

Creatine kinase is involved in energy transformation pathways. A sizable fraction of CK is present inside mitochondria (mi-CK) in addition to that present in sarcoplasm. The ATP synthesized inside the mitochondria is converted to phosphocreatine by mi-CK, later transported to sarcoplasm and reconverted to ATP by CK present in sarcoplasm. As CK and α-KGDH are susceptible to oxidative damage, the mi-CK and α-KGDH are more likely to be affected in vivo, due to its closeness to the site of ROS production i.e. present inside the mitochondria is the electron transport chain (ETC), a major source of free radicals.

In addition to mitochondrial ETC, xanthine oxidase is another important source of free radicals (Mekhfi *et al.*, 1996). Xanthine oxidase (XO) has the ability to produce hydrogen peroxide. *In vitro* studies have demonstrated that xanthine and XO had inhibitory effects on CK activity in heart tissues in a dose-dependent manner (Mekhfi *et al.*, 1996; Genet *et al.*, 2000).

In an anaerobic situation, LDH contributes to energy synthesis by anaerobic glycolysis. An increased production of ROS/RNS occurs during tissue hypoxia (Chen and Meyrick, 2004), which can negatively affect the activity of energy synthesis and transformation pathways. With hypoxia, activation of LDH enzyme by ROS may work as a force to counter the negative effect of other enzymes on energy synthesis and transformation pathways. Recently, we observed higher LDH activity in broilers developing CHF (Nain *et al.*, 2008b). Hence, increased activity of LDH in broilers developing CHF is most likely due to generated oxidative stress in the broilers. Insufficiency of creatine phosphate (CrP) and ATP leads to deterioration in heart pump function in broilers (Olkowski *et al.*, 2007a; Nain *et al.*, 2008b). However, the decline in these energy substrates could not be explained by changes in the end point kinetic activities of the rate limiting enzymes such as α-KGDH and PDH (Nain *et al.*, 2008b). In view of the present findings it is entirely possible that the inhibition of α-KGDH and

CK activity by oxidants can occur, regardless of their apparent kinetic activity *in vivo* (which may include compensatory up-regulation). This suggests that the observed decline in energy phosphates with deterioration in heart functions might be associated with the decreased activity of these enzymes during oxidative stress.

Our findings from lipid peroxidation assay strongly suggest that oxidative stress is generated during development of heart failure. In fast-growing broilers, oxidative damage is likely associated with excessive generation of H_2O_2. Maxwell *et al.* (1996) provides further supportive evidence as these authors demonstrated higher H_2O_2 activity in the cardiomyocytes from failing heart in broilers. However, as discussed above, several other enzymatic pathways may generate oxidants. Among the most relevant to oxidative damage in broiler heart is xanthine oxidase complex, which has very high activity in birds (Edson *et al.,* 1936). This may be biochemically related to specific features of nitrogen metabolism in fast-growing broilers as uric acid is the end product of purine metabolism in all vertebrates and in particular amino acid metabolism in birds. This reasoning is supported by the study of Enkvetchakul *et al.* (1993) who found that uric acid levels are higher in broilers with CHF, providing indirect evidence that XO may be involved in the pathogenesis of heart failure in broilers

Based on the findings from the present study, we conclude that the deterioration in heart pump function and ultrastructural lesions observed in mitochondria may be subsequent to oxidative stress, which affects mitochondrial energy generation and synthesis pathways leading to lowered energy reserve in the myocardium.

8. EFFECTS OF DIETARY VITAMIN E AND C SUPPLEMENTATION ON HEART FAILURE IN FAST-GROWING COMMERCIAL BROILERS

8.1. Abstract

Recently, we provided the proof of concept that oxidative stress is involved in the pathogenesis of congestive heart failure (CHF) in broiler chickens. Vitamins E and C, common antioxidants, have been advocated for the prevention of heart failure in broilers. Hence, in order to test the effects of supplementation of these vitamins on incidence of CHF and prevention of oxidative stress in myocardium, three experiments were conducted. Commercial male broilers were randomly allocated to three experimental groups, and respectively offered commercial broiler diet (control), commercial diet fortified with vitamin E (960 IU/kg) or vitamin C (400 mg/kg). The broilers were monitored daily for overt signs of heart failure and data including ECG and blood gas analysis were collected during 5^{th} week of experiment. Lipid peroxidation was measured in cardiac tissues from apparently normal broilers and broilers developing CHF in each group using thiobarbituric acid reactive substances (TBARS) assay. Overall, the incidence of CHF in broilers fed diet fortified with vitamin E was not significantly different as compared to the control group, whereas supplementation of vitamin C in diet tended ($p=0.10$) to reduce the incidence of CHF. The incidence of overt signs of hypoxemia based on clinical signs of cyanosis was lower ($p<0.05$) in vitamin C fed group as compared to the control group. The lipid peroxidation was highest ($p<0.05$) in broilers that developed CHF as compared to apparently normal broilers fed either vitamin E or C fortified diets. Neither vitamin E nor vitamin C was effective in preventing oxidative damage in broilers that developed CHF. In conclusion, the present study confirmed that oxidative stress is involved in the pathogenesis of heart failure in broilers, but dietary supplementation of antioxidant vitamins did not prevent oxidative damage in broilers that developed CHF. Beneficial effects of vitamin C supplementation were evidenced by lower incidence of hypoxemia,

and the tendency to reduce the susceptibility of broilers to heart failure. However, vitamin E did not have any impact on clinical status or the incidence of CHF.

8.2. Introduction

Previous studies from our laboratory (Olkowski *et al.,* 2001; Nain *et al.,* 2008b) revealed that congestive heart failure (CHF) in broilers is associated with extensive pathological changes in the ventricular myocardium, with distinctive lesions indicative of oxidative damage. Nain *et al.* (Unpublished) provided evidence that oxidative stress is an integral part of CHF pathogenesis.

Oxidative stress is associated with generation of several classes of radicals with high propensity towards oxidation of biological macromolecules such as lipids, proteins and nucleic acids. Reactive oxygen or nitrogen species (ROS/RNS) are the most common oxidants that play a critical role in development of human heart failure (Andreka *et al.,* 2004; Sam *et al.,* 2005; Nediani *et al.,* 2007). ROS and RNS can alter the properties of lipids, proteins and nucleic acids, leading to myocardial dysfunction (for review see Orrenius *et al.,* 2007).

Oxidative damage in the myocardium is associated with CHF. With regard to preventative measures of heart failure, substantial research interests have focused on vitamins E and C due to their purported ability to prevent oxidative damage. Historically, it has been commonly believed that the vitamin E supplements can lower risk of heart diseases in humans (Singal and Kirshenbaum, 1990; Bauersachs *et al.,* 2001). However, recent studies (Marchioli *et al.,* 2006; Robinson *et al.,* 2006) have questioned this tenet and some studies indicated that over-supplementation of vitamin E may actually increase the risk of heart failure (Yusuf *et al.,* 2000; Liu and Tan, 2002; Lonn *et al.,* 2005; Miller *et al.,* 2005; Shelton *et al.,* 2005). Similar to the findings in humans, contradictory reports exist about the use of vitamin E and C and their effects on the incidence of CHF in broilers. Some have suggested that vitamin E and/or C supplementation in broiler diet decreases the incidence (Al-Taweil and Kassab, 1990;

Bottje *et al.*, 1995) while findings of others suggested increased risk of CHF in broilers (Walton *et al.*, 2001; Lorenzoni and Ruiz-Feria, 2006).

The present study was designed to examine whether dietary supplementation of antioxidant vitamins (vitamins E and C) influences the incidence of heart failure and prevents oxidative damage in broilers developing heart failure. First, we investigated whether presence of these antioxidant vitamins in the broiler diet lowers the incidence of acute or chronic heart failure. This investigation included measurements of basic parameters of heart performance such as blood gas parameters and myocardial susceptibility to arrhythmia. In the second phase, we investigated the effect of these vitamins on the prevention of oxidative damage in apparently normal broilers and in those broilers developing congestive heart failure.

8.3. Materials and Methods

8.3.1. Experimental design, Animals and Management

Three experiments were conducted using commercial male broilers (Ross X Ross 308) randomly allocated into three experimental groups (for details see Table 8.1). Within each experiment, every experimental unit was replicated twice using separate pens, 40 to 50 broilers per pen. Male chicks were randomly assigned to six pens and offered a commercial broiler diet (vitamin E: 60 IU/kg of feed) or a commercial broiler diet fortified with vitamin E (960 IU/kg of feed) or vitamin C (400 mg/kg of feed) (DSM Nutritional Products, Canada). Feed and water were provided *ad libitum*.

Details of environmental conditions, management strategies and feeding regimes were as described previously (Olkowski *et al.*, 1999; Nain *et al.*, 2008b). Briefly, the broilers were housed in environmentally controlled room under constant light. During the first seven days, the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 30% (weeks 2, 3) and 40% (weeks 4, 5) lower than that recommended for normo-thermal brooding.

This lowered environmental temperature in fast-growing broilers results in increased metabolic rate. Higher metabolic demand for oxygen puts increased burden on the cardiovascular system and thereby precipitates heart failure in broilers predisposed to heart conditions.

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee. The procedures were performed as per the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

8.3.2. Clinical Monitoring

The standard experimental procedure (Chapter 3) followed during this study includes daily monitoring of broilers for overt signs of heart disease (fatigue, exercise intolerance, tachypnea, cyanosis and ascites). Broilers were diagnosed with CHF based on detailed clinical investigation for the above mentioned clinical signs and confirmed by postmortem findings.

Bluish discoloration of combs and wattles has been defined as cyanosis associated with hypoxemia, an indication of impaired cardiac function. In order to assess the proportions of broilers with cardiac dysfunction, the number of broilers showing cyanotic combs and wattles were counted in each treatment group. Evaluation of hypoxemia by cyanosis as a clinical parameter was validated by blood gas measurements. The blood gas analysis of broilers with cyanosis revealed higher pCO_2, lower pO_2 and lower hemoglobin O_2 saturation percentage as compared to apparently normal broilers.

8.3.3. Electrocardiographic Measurements

The data for heart rate, arrhythmia, and QRS axis deviation were collected using electrocardiography (ECG). Electrocardiographic records were obtained from 62 randomly derived apparently normal broilers from each group (10 to 15 from each pen) during the 5^{th} week of age. The ECG measurements were obtained as described

previously (Olkowski *et al.*, 1997). Briefly, ECG records were obtained using a lead II arrangement after induction of light anesthesia. The induction of anesthesia was accomplished with isoflurane (at a concentration of 2.0%) delivered by an agent-specific precision vaporizer in broilers inhaling pure (100%) oxygen at a flow rate of 1.0 L/min. Following induction, anesthesia was maintained with 1.0% isoflurane during ECG measurements. The signals from the ECG monitor were digitized using an analog to digital data recording unit and software (Mac Lab and Scope 3.3: AD Instruments Pty Ltd, Castle Hill, Australia) and processed using a Macintosh computer. The ECG data were evaluated for abnormal heart electrophysiological patterns and QRS axis deviation as described previously (Olkowski *et al.*, 1997).

8.3.4. Blood Gas Measurements

For blood gas measurements, approximately 0.5 mL blood samples were obtained anaerobically from the wing vein and analyzed for pH, pCO_2, pO_2, and hemoglobin O_2 saturation using a pH/Blood Gas Analyzer (Bayer Corporation, East Walpole, MA, USA). The samples were obtained from 7 randomly selected broilers from each pen at the end of the 5^{th} week of the experiment. Additionally blood samples were collected from 10 broilers showing signs of CHF.

8.3.5. TBARS Assay

Heart tissue samples were obtained from four randomly derived apparently normal broilers and four broilers with CHF from each group (random within pens) at the end of the experiment. These samples were collected following cervical dislocation and were snap frozen in liquid nitrogen.

Lipid peroxidation, an indicator of oxidative damage, was measured using malondialdehyde thiobarbituric acid reactive substances (TBARS) assay spectrophotometrically as described previously by Ohkawa *et al.* (1979) with some modifications. Briefly, frozen/pulverized heart tissue samples from mid portion of left ventricular myocardium were homogenized first in a KCl mixture (2 mM EGTA, 0.02% BHT in 1.15% KCl) using a microtube homogenizer and then RIPA buffer (50 mM

Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 1% sodium deoxycholate and 1% SDS, with pH adjusted to 7.2 using NaOH) was added and mixed. Finally 1 g of heart tissue was present in 6 mL of solution containing RIPA buffer and KCl mixture in a ratio of 4:5 v/v. The homogenate was centrifuged at 15000×*g* for 10 min at 4°C. 200 µL of supernatant was mixed with 0.2 mL 8.1% SDS, 2500 µL 30% acetic acid (pH adjusted to 3.5), 375 µL 0.8% TBA and 8.25 µL of 0.02% BHT aqueous solution followed by incubation at 95°C for 1h. After incubation and subsequent cooling, an equal volume of n-butanol/pyridine mixture (15:1 v/v ratio) was added. After vigorous shaking, it was centrifuged at 4000×*g* for 10 min. Following centrifugation, the organic layer obtained at the top was used for measuring thiobarbituric reactive substances at 532nm.

The calibration curve was prepared by using a group of five malondialdehyde (MDA) standards. Samples were diluted so that the expected concentration of the sample fell in the mid range of the calibration curve. The coefficient of determination was 0.99 for standard curve. The procedures showed high degree of precision and reproducibility, with inter assay coefficients of variation less than 5%.

8.3.6. Post Mortem Examination

Detailed gross post mortem examination was performed on broilers that died or were euthanized during the course of study and all the remaining broilers at the termination of the study. Sudden death syndrome (SDS) was diagnosed in well grown, apparently normal broilers that died suddenly without any other cause of death evident upon post mortem examination. The diagnosis of congestive heart failure (CHF) was based on gross dilation of the ventricular chambers along with abnormal accumulation of ascitic fluid in abdominal cavity.

8.3.7. Light Microscopy

The hearts from three randomly selected broilers within each group and three birds with CHF were processed for histopathological examination. The samples were fixed in buffered formalin and subsequently blocks of myocardium were embedded in

paraffin. Sections (5 μm thickness) were processed for light microscopy and stained with hematoxylin/eosin.

8.3.8. Statistical Analysis

Data were analyzed using the microcomputer package Number Cruncher Statistical System (Hintze, 1995). Lipid peroxidation and blood gas data were analyzed using analysis of variance and means were separated by using Fisher's LSD. Incidence of CHF, SDS and descriptive clinical, and ECG data were analyzed using Fisher's exact test. Incidence of CHF, SDS and descriptive clinical data were analyzed using data combined from all these three experiments. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

8.4. Results

Overall, the incidence of fulminant CHF tended to be lower ($p = 0.1$) in broilers fed vitamin C (35.1%) as compared to the control group (42.0%) (see Table 8.1). The incidence of CHF in the group fed vitamin E (41.0%) was not different ($p>0.05$) from the control group. The relative risk (ratio of the proportions of cases) of overall mortality/morbidity associated with heart failure (CHF and SDS) in the present study in broilers fed the vitamin E or vitamin C fortified diet were 1.02 and 0.87 respectively, in comparison to broilers fed the control diet.

Table 8.1. Incidence of chronic heart failure (CHF) and sudden death syndrome (SDS) in broilers fed the control (CTR) diet and those fed the diet supplemented with vitamin E or C.

	Experimental Group	CHF (Mortality/Morbidity)	SDS (Mortality)
Exp. 8.1, 8.2 & 8.3	CTR (n=288)	121/288† 42.0%‡	13/288 4.5%
	Vit. E (n=288)	118/288 41.0%	19/288 6.6%
	Vit. C (n=288)	101/288 35.1%	16/288 5.6%
Significance (Fisher's Exact Test)	CTR, Vit. C	p = 0.10	p = 0.70
	CTR, Vit. E	p = 0.87	p = 0.36

† Frequency of occurrence; ‡ % of total broilers; n = number of broilers.

Measurement of the incidence of cyanosis at day 32 from all the experiments revealed that the percentage of broilers with cyanotic combs and wattles was lower ($p<0.05$) in the group fed the vitamin C supplemented diet (28.0%) as compared to the control group (40.0%) or the group fed the vitamin E supplemented diet (40.3%).

Blood gas measurements showed that broilers with CHF had severe hypercapnia (pCO_2: 57.7 mmHg), hypoxemia (pO_2: 22.1 mm Hg) and the lowest hemoglobin O_2 saturation (HbO_2Sat: 33.8 %) as compared to apparently normal broilers fed the control diet or the diets supplemented with vitamin E or C (Table 8.2). However, there were no significant ($p>0.05$) differences in broilers pCO_2, pO_2 and HbO_2 saturation % between apparentally normal broilers fed the control diet as compared to the group fed with vitamin E or C fortified diet. There was no difference ($p>0.05$) in the heart rate (HR) in broilers fed with either vitamin C fortified diet (HR: 328 beats/minutes) or vitamin E fortified diet (321 beats/minutes) or the control diet (322 beats/minutes).

Table 8.2. Blood gas profiles and heart rate from randomly derived broilers in group fed the control (CTR) diet and those fed the diet supplemented with vitamin E or C

	pH (Units)	pCO_2(mm Hg)	pO_2(mm Hg)	Hb O_2 Saturation %	Heart Rate (beats/min)
CTR	7.42 ± 0.014^b	49.2 ± 1.78^a	33.1 ± 1.18^b	62.7 ± 2.8^b	322 ± 3.8^b
Vit E	7.40 ± 0.015^b	46.3 ± 1.90^a	33.0 ± 1.22^b	61.9 ± 2.96^b	321 ± 3.9^b
Vit C	7.42 ± 0.010^b	44.5 ± 1.40^a	35.5 ± 1.10^b	67.4 ± 2.44^b	328 ± 3.5^b
Broiler-CHF	7.34 ± 0.022^a	57.7 ± 3.97^b	22.1 ± 1.27^a	33.8 ± 3.78^a	231 ± 9.4^a
P Value	$p<0.01$	$p<0.01$	$p<0.001$	$p<0.0001$	$p<0.0001$

Values are means ± SE (n=21 for CTR, Vit E and Vit C; n=10 Broiler with CHF). Means within column with different superscripts are significantly different ($p<0.05$).

In order to understand the trends in blood gas parameters (pCO_2, pO_2 and Hb O_2 Saturation %) and heart rate in broilers fed with different dietary sources, and from broilers with CHF were expressed with reference to levels in broilers fed the control diet taken as 100%. These comparisons are presented in Figure 8.1.

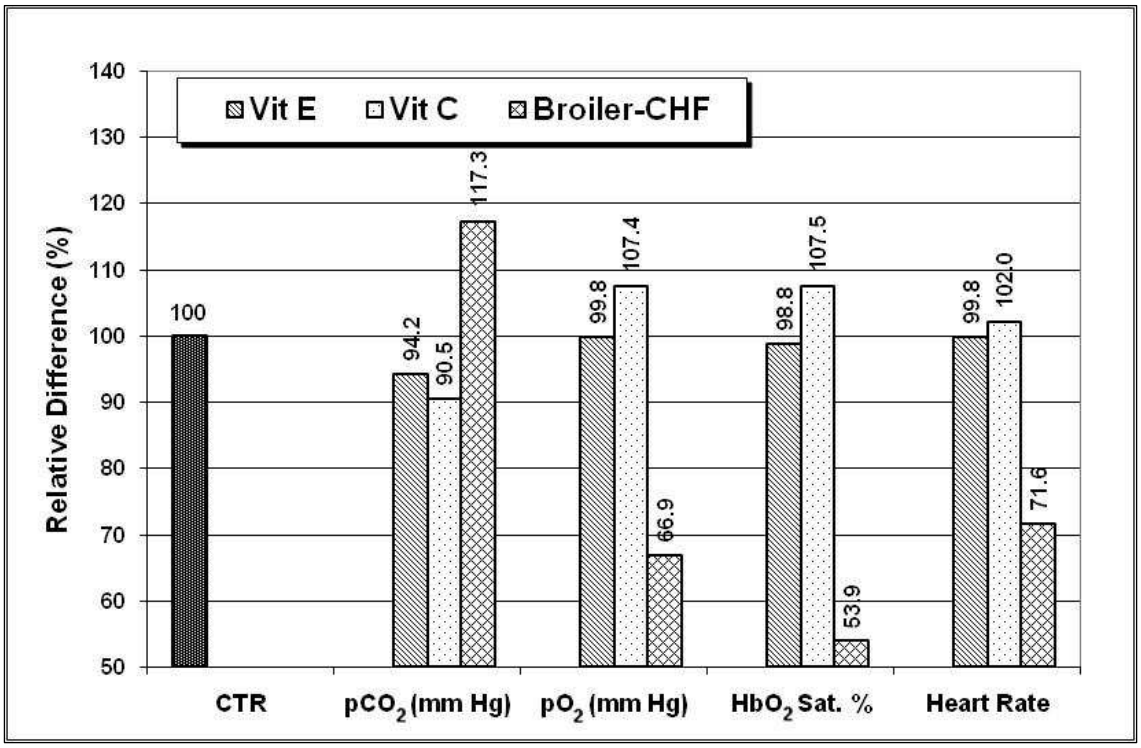

Figure 8.1. Relative levels of blood gas parameters (pCO_2, pO_2 and HbO_2 saturation %) and heart rate in the vitamin E fed group (Vit E), vitamin C fed group (Vit C) and in broilers with congestive heart failure (Broiler-CHF) expressed on percentage basis of broilers fed the control diet (CTR) representing on the graph a reference value of 100.

Negative QRS axis (consistent with dilated cardiomyopathy) was observed in 41.1 % of the broilers in the control group, while in the groups fed diets with vitamin E or vitamin C negative QRS axis was observed in 53.6% and 26.8% of broilers, respectively. There was no difference ($p>0.05$) incidence of cardiac arrhythmia in broilers fed with either vitamin C fortified diet (10.7%) or vitamin E fortified diet (19.6%) or the control diet (14.3%).

The post mortem examination of broilers that developed CHF during the course of study revealed abnormal dilation of atrial and ventricular chambers, nodular thickenings on the edges of atrio-ventricular valve, pericardial effusions and abnormal accumulation of ascitic fluid in abdominal cavity.

MDA equivalent values, an indicator of lipid peroxidation, were highest ($p<0.05$) in broilers that developed CHF irrespective of the dietary treatment as compared to apparently normal broilers fed with either vitamin E or C fortified diet (see Figure 8.2). The presence of vitamin E or C in broilers diet numerically lowered lipid peroxidation in apparentally normal broilers as compared to the broilers in control group, but there were no significant differences ($p>0.05$).

Histo-pathological examination revealed degenerative changes in CHF broilers and in some apparently normal broilers from all three experimental groups, but there were considerable differences in the magnitude of the lesions (Figure 8.3). The lesions were more extensive in myocardium of broilers fed either the control diet or the diet supplemented with vitamin E as compared to broilers fed vitamin C.

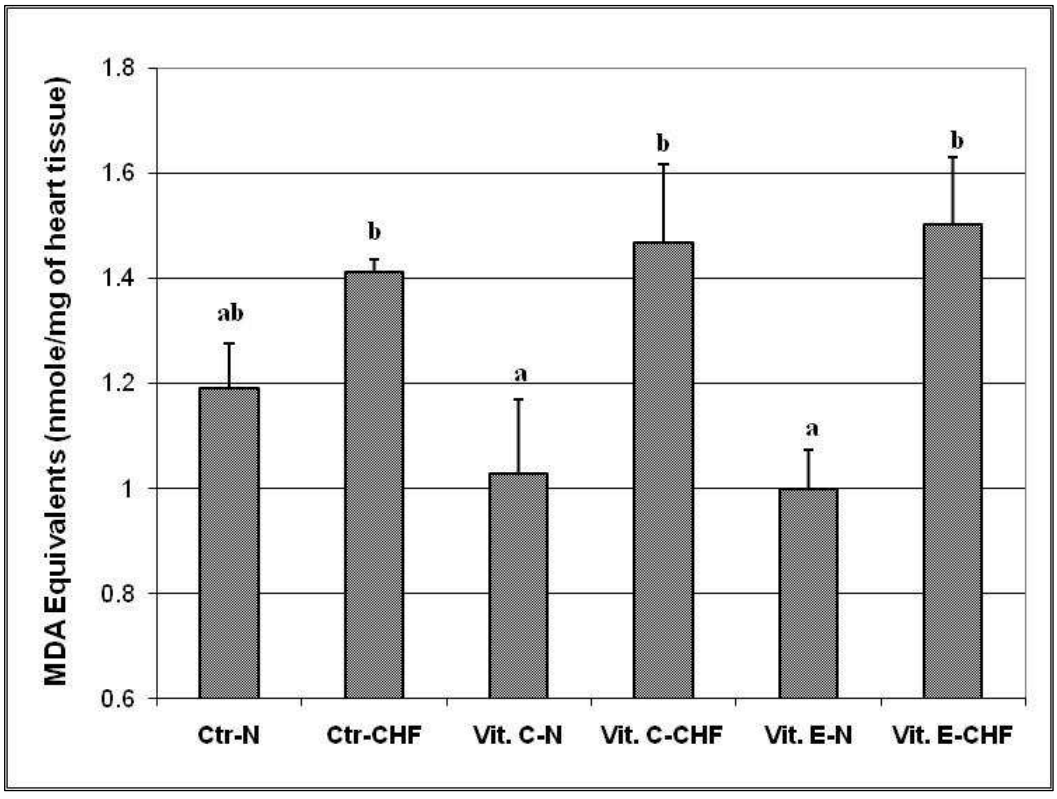

Figure 8.2. Effects of vitamin E and C supplementation effect on lipid peroxidation in apparently normal broilers (CTR-N, VIT C-N and VIT E-N) and in those with congestive heart failure (CTR-CHF, VIT C-CHF and VIT E-CHF) as measured using thiobarbituric acid reactive substance (TBARS) assay.
[a,b] Bars with different superscripts are significantly different ($p< 0.05$).

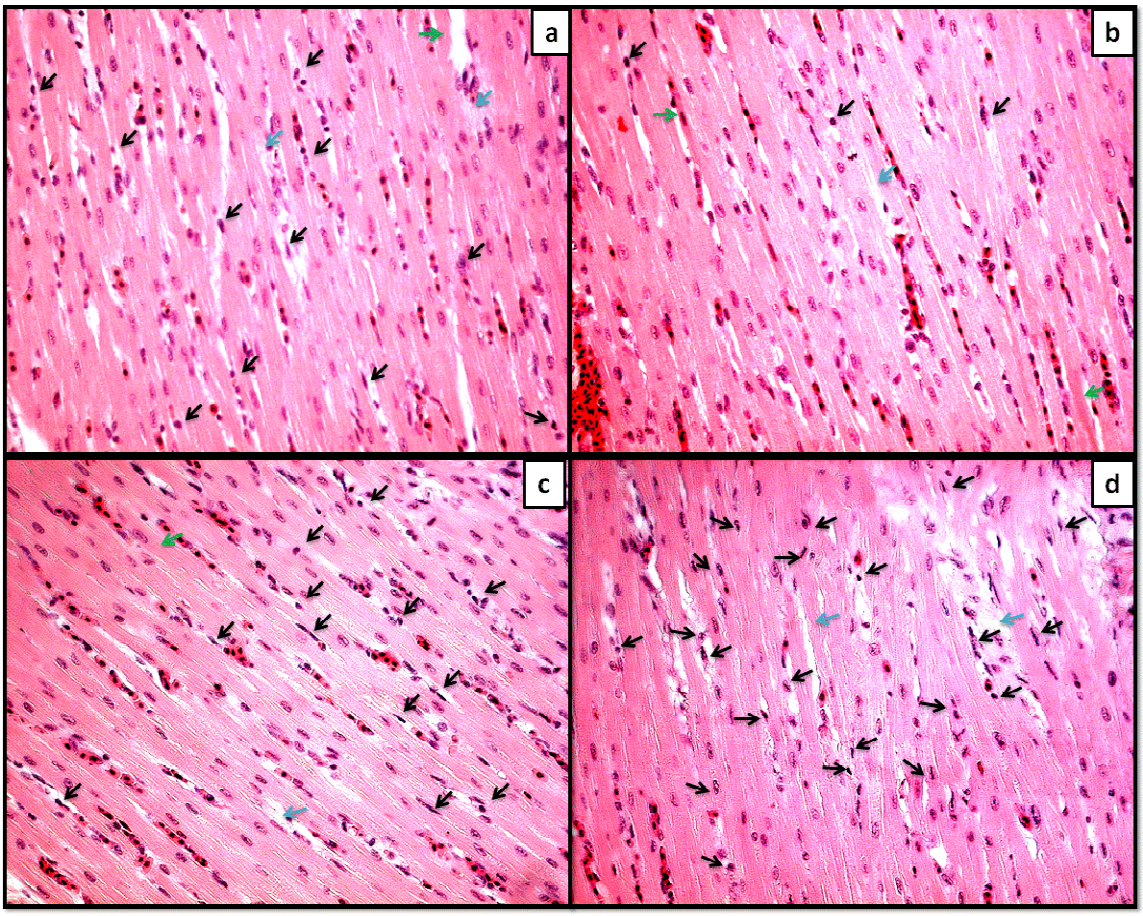

Figure 8.3. Representative histo-pathological features from mural left ventricular myocardium of a randomly selected broiler from the group fed the control diet (a), the diet supplemented with vitamin C (b) or vitamin E (c) and from a broiler that developed congestive heart failure (d). Original magnification 400X).
The observed ventricular myocardium lesions consisted of degenerative changes in the cardiomyocytes characterized by nuclear pyknosis, karyorrhexis and degenerative changes in nuclei indicative of cell death (black arrows). Cytoplasmic eosinophilia (green arrow) observed in some cardiomyocytes, an early indicator of cellular changes subsequent to myocardial injury. Additionally, some cardiomyocytes appears wispy pale and loosing typical banding pattern, indicative of loss and thinning of myofibrillar components (blue arrow), in advanced cases cardiomyocyte dropout can be observed. The lesions have similar qualitative features in all the three groups, but the degenerative changes were more extensive in broilers fed the control and the vitamin E fortified diets as compared to broilers fed the vitamin C fortified diet. The most severe and extensive lesions were observed in broilers that developed congestive heart failure.

8.5. Discussion

The present study confirmed that oxidative stress is involved in the pathogenesis of heart failure in broilers, but neither of the antioxidant vitamins tested in this study was effective in preventing lipid peroxidation in broilers that developed CHF. Interestingly, our findings revealed that supplementing vitamin C in broilers diet may have some beneficial effect in preventing congestive heart failure as revealed by clinical, mortality/morbidity and histo-pathological findings, whereas vitamin E supplementation provided no beneficial effect in preventing heart failure in broilers.

Our findings on vitamin E are consistent with earlier reports that supplementation of vitamin E in broilers diet has no favorable outcome or even increases the risk of heart failure (Bottje *et al.*, 1997; Walton *et al.*, 2001; Lorenzoni and Ruiz-Feria, 2006). However, this contradicts other findings that vitamin E prevents CHF in broilers (Bottje *et al.*, 1995; Kawthekar, 2007).

Beneficial effects of vitamin C were reported by others (Al-Taweil and Kassab, 1990; Ladmakhi *et al.*, 1997; Xiang *et al.*, 2002). In our study, the relative risk of heart failure (RR=0.87) in broilers fed the vitamin C diet tended to be lower in comparison to the control. Additionally, supplementation of vitamin C resulted in lowered incidence of cyanosis. In broilers cyanosis has been found to be associated with hypoxaemia and increased risk of congestive heart failure (Olkowski *et al.*, 2005b; Nain *et al.*, 2008b). However these findings on incidence of cyanosis were not clearely supported by the blood gas analysis.

One of the new and interesting observations from the present study is that presence of vitamin C or E in broilers diet was unable to prevent lipid peroxidation in broilers that developed CHF. It is interesting to mention here that life span of free radicals is very short and they cause oxidative damage before being trapped by antioxidants.

Recently, we observed that oxidative stress in the myocardium is involved in the pathogenesis of CHF in broilers (Nain *et al.*, Unpublished). The present findings raise doubt about the effectiveness of vitamins E and C in the prevention or reduction of this oxidative stress in myocardium of broilers developing CHF.

Vitamin E acts as antioxidant by preventing the oxidation of polyunsaturated fatty acids in cellular and sub cellular membranes and decreases systemic oxidative stress (Hidiroglou *et al.*, 2004; Roberts *et al.*, 2007), however its effects are more likely to be restricted to lipid components inside the cell due to its chemical nature. Creatine kinase (CK) located in the mitochondrial matrix and cytoplasm is involved in energy transformation pathways inside the myocardium. The α-ketoglutarate dehydrogenase (α-KGDH), one of the rate limiting enzymes of the tri-carboxylic acid (TCA) cycle is present in mitochondrial matrix. Both of these enzymes are susceptible to oxidative stress in broilers (Nain *et al.*, 2008a). Hence the potential ability of vitamin E to prevent oxidative damage of these enzymes is less likely due to the difference in cellular locations of these enzymes and vitamin E.

It is commonly assumed that broilers do not require vitamin C from dietary sources due to their innate ability to synthesize it. Therefore, its supplementation in broiler diets is not practiced. However, certain environmental or pathological conditions may increase its requirements beyond the capacity to synthesize (Pardue and Thaxton, 1986). L-ascorbic acid acts as an antioxidant and a co-factor for enzymes involved in the synthesis of collagen, catecholamines and carnitine (Hulse *et al.*, 1978; Seitz *et al.*, 1998; Naidu, 2003). Recently, we observed lowered carnitine levels in broilers developing CHF (Nain *et al.*, 2008b). However, it needs to be established that the favorable effects of vitamin C observed in the present study are a consequence of its ability to prevent oxidative stress or ability to work as a co-factor for enzymes involved in the synthesis of collagen, catecholamines and carnitine. Previous studies have reported a lower level of thyroxine with vitamin C supplementation (Ladmakhi *et al.*, 1997), a hormone implicated with increased risk of CHF in broilers (Scheele *et al.*, 1992; Decuypere *et al.*, 1994; Malan *et al.*, 2003). Hence, here beneficial effects of

vitamin C supplementation observed in the present study may be due to its antioxidant property or due to its ability to work as cofactor, and further exploration is justified.

The present findings suggest that vitamin E supplementation is not effective in preventing CHF in broilers, although it appears to prevent oxidative damage in normal broilers. However, the study provides some evidence that vitamin C supplementation may have a beneficial effect by improving heart function and preventing CHF in broilers.

9. BIOCHEMICAL FACTORS LIMITING MYOCARDIAL ENERGY IN A CHICKEN GENOTYPE SELECTED FOR RAPID GROWTH

9.1. Abstract

In comparison to slow-growing broilers, many fast-growing broilers show signs of impaired heart function manifested as fatigue, exercise intolerance, tachypnea, and cyanosis. In more advanced cases some of these birds develop clinical signs of congestive heart failure (CHF) and ascites. Recently it was demonstrated (Olkowski *et al.*, 2007a) that the deterioration of heart function is associated with inadequacy of chemical energy substrate in the ventricular myocardium. In order to further understand the etiological mechanisms associated with the poor performance of the heart pump and development of CHF in broiler chickens, this work examined heart function, blood gas parameters, and status of several factors critical for sustainable myocardial energy metabolism in feed restricted slow-growing broilers (low risk of heart failure), fast-growing broilers (high risk of heart failure), and broilers with CHF. Measured variables included cardiac levels of substrates in energy metabolism [creatine phosphate (CrP), adenosine triphosphate (ATP), and L-carnitine] and activity of selected cytosolic enzymes [creatine kinase (CK), and lactate dehydrogenase (LDH)] and mitochondrial enzymes [pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH)]. In comparison to slow-growing broilers, heart function in fast-growing broilers and in broilers with CHF was impaired as evidenced by lower heart rate, blood pO_2, Hb O_2 saturation % and higher pCO_2 levels. Broilers with CHF had a lower myocardial energy reserve pool (CrP and ATP) and L-carnitine level (all $p<0.05$). The CK activity was higher ($p<0.05$) in *ad libitum* fed and CHF broilers as compared to feed restricted slow-growing birds. The PDH activity was highest ($p<0.05$) in broilers with CHF. The LDH and α-KGDH activities were not different ($p>0.05$) among various categories of birds.

Our findings indicate that deterioration of heart function associated with rapid growth is correlated with insufficiency of the cardiac energy reserve pool (ATP and CrP). Lower levels of myocardial L-carnitine indicate impaired delivery of fatty acids (FA) to heart mitochondria for energy synthesis. Since the FA β-oxidation pathway accounts for the majority of myocardial ATP synthesis, insufficiency of FA substrate for this pathway is likely a major reason for the low energy reserve pool in cardiomyocytes, thereby contributing to deterioration of heart function. In slow-growing broilers, cardiac ATP synthesis appears to be sufficient to sustain normal heart function, but in fast-growing broilers and in broilers with CHF, cardiac energy management also depends on ATP regeneration from CrP as evidenced by increased CK activity and decreasing levels of CrP. Depletion of CrP is likely a major cause of progressing ATP deficiency and further deterioration of heart function, which in more advanced cases results in congestive heart failure.

9.2. Introduction

In broilers genetic selection is primarily focused on rapid weight gain and feed conversion efficiency. This has resulted in broiler strains that have superior performance characteristics for traits of economic importance (weight gain and feed conversion efficiency), but also higher incidences of specific physiological insufficiencies predisposing many birds to health problems. Heart failure is one of the most prominent systemic weaknesses commonly seen in commercial broiler flocks.

With the high metabolic demand associated with rapid growth, heart function in many broilers is marginally capable of providing enough blood flow to sustain the basic oxygen requirements. Studies from our lab revealed that a relatively large number of commercial broilers show evidence of hypoxemia associated with sub-clinical heart disease (Olkowski *et al.*, 1999; 2005a).

Various clinical observations such as blood gas analysis, echocardiography, electrocardiography, and necropsy findings obtained from several cross-sectional studies indicate that a large population of broilers are either at risk of heart failure with

various sub clinical problems or showing signs of heart failure (Olkowski *et al.,* 2003b; Olkowski, 2007a).

Fast-growing broilers have an inherent predisposition towards increased risk of heart failure (Navarro *et al.,* 2006; Druyan *et al.,* 2007). However, this problem does not appear to be associated with any genetic defect *per se*, but rather with a metabolic limitation of the myocardium. The rapid growth rate predisposes broilers to heart failure, but when growth rate is controlled by dietary restriction, heart function is improved and the risk of heart failure decreases significantly (Olkowski and Classen, 1998b; Olkowski *et al.,* 2005a; 2007a).

In comparison to breeds of chickens not selected for growth such as leghorns, fast-growing broilers have relatively smaller structural and functional hearts, and reduced capacity of the left ventricle (Martinez-Lemus *et al.,* 1998; Olkowski *et al.,* 2005b). The cardiac index (ml of blood ejected by ventricles / kg body weight / minute), an indicator of heart performance is lower in fast-growing broilers and broilers with congestive heart failure (CHF) as compared to leghorn chickens or feed-restricted broilers (Olkowski *et al.,* 1999), suggesting impaired heart function in fast-growing broilers. Broilers with CHF show lowered left ventricular fractional shortening, which indicates impaired contractile functions in the heart (Olkowski *et al.,* 2005a; Deng *et al.,* 2006).

The most characteristic early patho-physiological feature of deteriorating heart pump function in fast-growing broilers is a declining heart rate (Olkowski and Classen, 1998b; Deng *et al.,* 2006; Druyan *et al.,* 2007). Energy depletion in failing myocardium has been documented in animal and human research (for review see Stanley *et al.,* 2005). Recent study identified depletion of high energy phosphates [creatine phosphate (CrP), adenosine triphosphate (ATP)] as a major factor associated with deterioration of heart function in broilers (Olkowski *et al.,* 2007a). Hydrolysis of ATP provides energy required for mechanical work of the heart muscle, while CrP act as an energy reserve in the myocardium.

The molecular mechanisms underlying the changes in cardiac energy metabolism in fast-growing broilers are not clear. Therefore in order to better understand metabolic events associated with changes in myocardial energy generation pathways, we investigated the status of selected critical parameters involved in high energy phosphate metabolism with emphasis on rate limiting substrates [CrP, ATP and L-carnitine] and activity of selected cytosolic enzymes [creatine kinase (CK), and lactate dehydrogenase (LDH)] and mitochondrial enzymes [pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (α-KGDH)]. These parameters were examined in feed restricted slow-growing broilers (low risk of heart failure), *ad libitum* fed fast-growing broilers (high risk and incidence of heart failure), and broilers with clinical signs of congestive heart failure (CHF). Reported studies indicate that slow-growing feed-restricted broilers show low incidence of heart failure (Olkowski and Classen, 1998b; Olkowski *et al.,* 1999; 2005b), therefore we used these birds as physiological reference.

9.3. Materials and Methods

9.3.1. Animals and Management

Chickens used in the present study represent a genotype of a typical commercial broiler. A total of 122 day-old male commercial broilers (Ross X Ross 308) were obtained from a local hatchery. The management protocol used in this study has been routinely used in our laboratory (Olkowski *et al.,* 1999; 2005b; 2007a). Briefly: All chickens were offered commercial broiler diet, and had unrestricted access to water. From day 1 to day 7, they were raised in a common pen and were fed *ad libitum*. On day 7, the chickens were randomly allocated to two experimental groups. Group1, designated as fast-growing consists of 83 birds, were randomly allocated to two pens and and fed *ad libitum* throughout the study, whereas Group 2, consisting of 39 birds, designated as slow-growing was subjected to a feed restriction regime kept in a separate pen. The chickens in the feed-restricted group were fed daily with a diet equal to 70% of the *ad libitum* fed group, from day 7 until the end of experiment (six week of age).

The birds were housed from day old in an environmentally controlled room under constant light. During the first seven days the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 30% (weeks 2 and 3) and 40% (weeks 4 and 5) lower than that set for normo-thermal brooding.

The management model used in the present study has been extensively tested and validated. This approach is very useful in studies on the physiology of heart function in chickens genetically selected for rapid growth. The lowered environmental temperature forces the birds to increase their metabolic rate, which results in an increased burden on the cardiovascular system. This is very effective in precipitating heart failure in all broilers predisposed to heart conditions, but only if they are fed *ad libitum.* The feed restriction regime results in slower growth, with body weight lowered by 25 to 30% in feed restricted group than that of ad-libitum fed broilers, and this slower growth rate reduces the risk of heart failure in these birds practically to zero.

Experimental protocols were approved by the University of Saskatchewan Animal Care Committee and procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

9.3.2. Clinical Evaluation

A standard experimental procedure was followed throughout the study with daily monitoring of overt clinical signs of heart failure (fatigue, exercise intolerance, tachypnea, cyanosis, and ascites). Birds showing signs of congestive heart failure (CHF) such as tachypnea, cyanosis and ascites were euthanized, and subjected to post mortem examination. Birds were visually assessed for presence of any sign of CHF. If showing signs, then these were classified as birds with CHF and subsequently data were collected from birds with CHF, apparently normal birds within *ad libitum* fed group, and the feed restricted group.

9.3.3. Post Mortem Examination

Detailed gross post-mortem examination was performed on all mortalities, euthanized birds, and all remaining birds upon termination of the study. The diagnosis of CHF was based on gross dilation of the ventricular chambers, pulmonary congestion, and abnormal accumulation of ascitic fluid in abdominal cavity.

9.3.4. Transmission Electron Microscopy (TEM)

To determine the ultrastructural changes associated with fast growth, hearts from three *ad libitum* fed broilers were processed for TEM. Heart tissue samples from the mid portion of the left ventricular wall were processed as described previously (Olkowski *et al.,* 2001).

9.3.5. Heart Rate, Respiratory Rate, and Blood Gas Measurements

The heart rate and respiration rate measurements were obtained from fifteen randomly selected birds from each category (feed restricted, ad libitum fed, and birds with CHF) during the fifth week of the experiment. Heart rate was measured using electrocardiography (ECG) as described previously (Olkowski and Classen, 1998a). The respiration rate was measured in unrestrained birds, as restraining affect their respiration rate because birds become distressed when handled. Undoubtedly this would not be physiologically acceptable measurement. Therefore, In order to avoid possible artifactual effects of stress associated with handling we devoted lot of time to record these data from unrestrained birds. Technically, this was done in such a way that investigator monitored the abdominal movement associated with respiration in the individual birds for a period of time sufficient to obtain reproducible results. Average of three measurements was recorded.

Blood gas measurements were obtained at the end of the fifth week of the experiment. For blood gas measurements, approximately 0.5 mL blood samples from wing vein were obtained anaerobically from 10 randomly selected birds from each group, and 10 birds with CHF. The samples were analyzed for pH, pCO_2, pO_2 and

hemoglobin O_2 saturation percentage using a pH/Blood Gas Analyzer (Bayer Corporation, East Walpole, MA, USA).

9.3.6. High Energy Phosphates Measurements

Creatine phosphate (CrP) and adenine triphosphate (ATP) were measured in left ventricular myocardial tissue of five randomly selected birds from each category (slow-growing feed-restricted broilers, fast growing *ad libitum* fed broilers and broilers with CHF) using an HPLC method (Olkowski *et al.*, 2007a). The method was validated for precision, accuracy and analyte recovery. The coefficient of determination for calibration curves for these analytes approached 0.999. The extraction of analytes from the samples was found to be complete as after first extraction, subsequent extractions of the processed samples did not yield any measurable amount of the analytes. This procedure showed a high degree of precision and reproducibility.

9.3.7. Enzyme Activity Assays

We focused on two cytosolic [Creatine Kinase (CK) and Lactate Dehydrogenase (LDH)] and two mitochondrial [Pyruvate Dehydrogenase (PDH) and alpha-Ketoglutarate Dehydrogenase (α-KGDH)] enzymes involved in energy synthesis and transformation pathways. The activities of these enzymes were measured in heart tissue obtained from the mid portion of the left ventricle free wall, using five samples in each group. Briefly, aliquots of 300 mg of frozen samples were homogenized in phosphate buffer (50mM, pH 7.4) in the ratio of 100 mg tissue per 1 mL buffer in test tubes pre-cooled with ice. The homogenate was centrifuged at 2,500×g for 10 min at 4°C and the suspension was further centrifuged at 12,000×g for 10 min. The supernatant was used for CK and LDH measurements. The CK activity was measured using a CK kit (Roche Diagnostics, Indianapolis, IN, USA) while LDH was measured using LD-L10 (Sigma Diagnostics Inc., St. Louis, MO, USA). The mitochondria containing pellet was further washed three times to remove remnants of cytosolic enzymes and finally this pellet was re-suspended in Tris buffer (50 mM, pH 7.6 with 0.5% Triton). PDH and α-KGDH activities were assayed in mitochondrial extracts. PDH activity was measured as described by Chiang and Sacktor (1975) with minor modifications where the final

components of the incubation media were 2 mM pyruvate, 2.5 mM NAD, 0.15 mM flavin adenine dinucleotide, 2 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.13 mM Coenzyme A, 2.6 mM dithiothreitol, and 30 mM Tris buffer at pH 7.2. The α-KGDH activity was measured in principle as described before (Olkowski and Classen, 1999) with modifications, where the final components of the incubation media were 3.2 mM α-keto glutaric acid, 2 mM NAD, 0.5mM Coenzyme A, 0.7 mM thiamine pyrophosphate, and 1 mM $MgCl_2$. The assays were validated for the linearity of responses for time of reaction and protein content. The PDH and α-KGDH assays were assessed for dependence of various cofactors present in the reaction mixture during validation phase. The reactions were found to be complete in presence of all the cofactors in the reaction cocktail. The reaction was started by adding 20 µL of cytosolic or mitochondrial fraction in 200 µL of cocktail per well in a 96 well micro plate pre incubated at 37°C for all these enzymes. Enzyme activity measurements were performed at 340 nm using a microplate reader SpectraMax Plus (Molecular Devices, CA, USA). The final activity measurements were performed during the linear phase of responses pre-established during validation phase. These measurements were performed in a single assay for each enzyme to avoid inter assay variability.

9.3.8. L-Carnitine Analysis

L-Carnitine was measured from the mid-portion of the left ventricle using an HPLC method (Feng *et al.,* 2006) with minor modifications. Briefly, four samples from the mid portion of left ventricles from each bird weighing 500-600 mg were homogenized with phosphate buffer (50 mM, pH 7.4) in the ratio of 200 mg tissue : 1 mL buffer. The homogenate was centrifuged at 2,500×g for 10 min at 4°C. The supernatant was precipitated using acetonitrile and methanol (9:1 v/v). A 300 mg mixture of Na_2HPO_4 and Ag_2O (9:1 wt/wt) and 300 mg of KH_2PO_4 were added followed by a one hour vortex. Derivatizing reagent (40 mg/mL p-bromophenacyl bromide with 50 µL 40% tetrabutylammonium hydroxide) was added into the organic extract. The reaction mixture was incubated at 60°C for 2 hours followed by centrifugation at 12,000×g for 15 min. L-carnitine was analyzed using a HPLC system (Agilent 1050) with Hyperclone 5 µ CN analytical column (Phenomenex, CA, USA).

The mobile phase (90% acetonitrile/10 mM citric-phosphate buffer, adjusted to pH 3) was delivered at a flow rate of 1 mL/min. The elution of carnitine was monitored at 260nm.

9.3.9. Statistical Analysis

Statistical analyses were carried out by GLM ANOVA from the microcomputer package Number Cruncher Statistical System (Hintze, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

9.4. Results

The first cases of CHF occurred during the 3rd week, and were observed onwards until the termination of experiment. At day 32 of the experiment, out of 62 surviving birds in the *ad libitum* fed group, 37 developed cyanosis, an early sign of heart failure. No signs of cyanosis were observed in the feed restricted group. Overall, during the course of this study, CHF (based on mortality and morbidity data) was observed in 38 out of 83 (46%) broilers from the *ad-libitum* fed group. None of the broilers from the feed restricted group showed clinical signs indicative of heart disease, and no cases of heart failure were observed on post mortem examination at termination of the experiment in the feed restricted group.

Heart and respiration rate measurements differed significantly ($p<0.05$) among the two treatment groups, and in broilers with CHF (Figure 9.1). The cardiac and respiratory measurements revealed a marked ($p<0.05$) decline in heart rate (HR: 308 beats/min) and an elevated respiration rate (RR: 57 breaths/min) in the *ad libitum* fed group as compared to the feed restricted group (HR: 371 beats/min; RR: 51 breaths/min). Profound bradycardia and tachypnea (HR: 236 beats/min and RR: 70 breaths/min) were observed in birds with fulminant CHF.

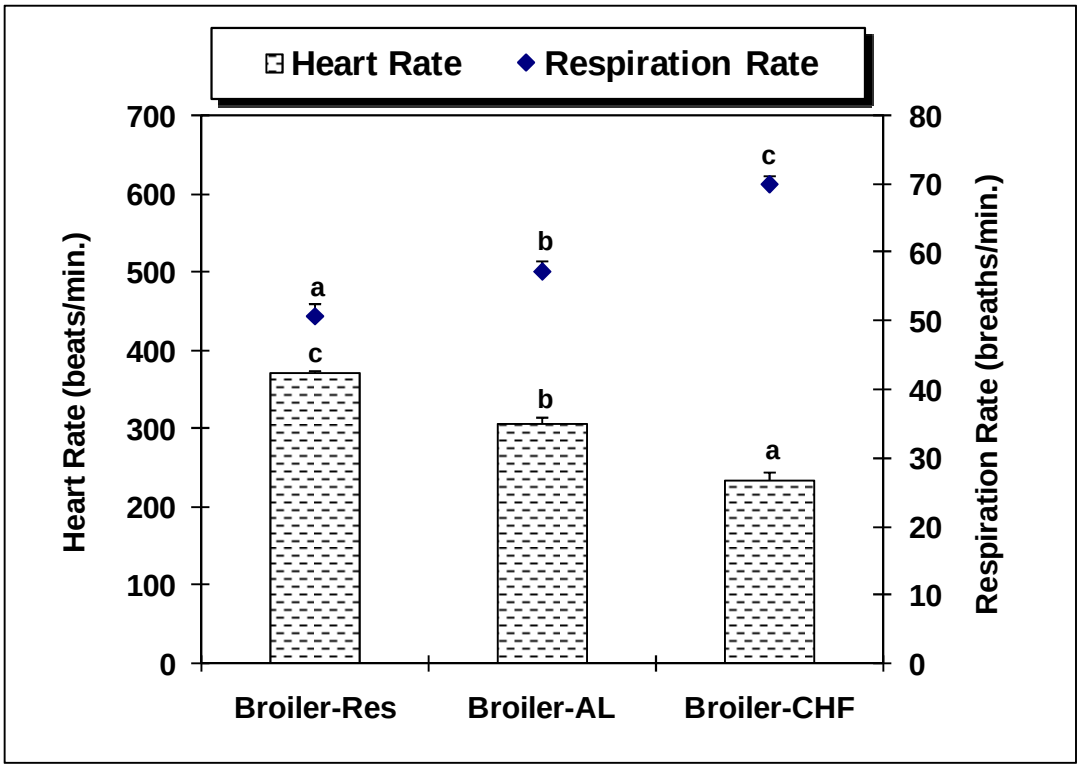

Figure 9.1. Heart rate and respiration rate in feed restricted slow-growing broilers (Broiler-Res), fast-growing broilers fed *ad libitum* (Broiler-AL), and in broilers with congestive heart failure (Broiler-CHF, observed within *ad libitum* fed group). [ab] Adjacent bars with different superscripts are significantly different ($p<0.05$); Values are means ± SE (n=15).

The blood gas measurements revealed marked hypercapnia (pCO_2: 48.3 mm Hg), hypoxemia (pO_2: 38.2 mm Hg) and lower hemoglobin oxygen saturation (Hb O_2 saturation %: 69.3%) in the *ad libitum* fed group as compared to the feed restricted group (pCO_2: 36.4 mm Hg; pO_2: 41.9 mm Hg; and Hb O_2 saturation 71.2%). Birds with CHF showed severe hypercapnia (pCO_2: 58.1 mm Hg), hypoxemia (pO_2: 21.3 mm Hg) and the lowest hemoglobin O_2 saturation (30.6%) as compared to feed restricted slow-growing and *ad libitum* fed fast-growing birds (Table 9.1).

Table 9.1. Comparison of blood gas parameters in feed restricted slow-growing broilers (Broiler-Res, low risk of heart failure), *ad libitum* fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with congestive heart failure and ascites (Broiler-CHF).

Parameter	**Broiler-Res**	**Broiler-AL**	**Broiler-CHF**	**P Value**
pH (Units)	7.40 ± 0.022^{b}	7.39 ± 0.021^{b}	7.31 ± 0.018^{a}	*p<0.012*
pCO_2 (mm Hg)	36.4 ± 1.46^{a}	48.3 ± 2.52^{b}	58.1 ± 2.63^{c}	*p<0.001*
pO_2 (mm Hg)	41.9 ± 1.56^{b}	38.3 ± 2.47^{b}	21.3 ± 1.19^{a}	*p<0.001*
Hb O_2 Saturation %	77.2 ± 2.12^{b}	69.3 ± 5.45^{b}	30.6 ± 2.86^{a}	*p<0.001*

Values are means ± SE from 10 birds sampled for blood gas analysis from each group. Means within rows with different superscripts are significantly different ($p < 0.05$).

Post mortem examination in all birds showing signs of CHF revealed abnormal accumulation of ascitic fluid in abdominal cavity along with gross dilation of the ventricular chambers. Ultrastructural examination revealed that cardiomyocytes frequently contained large aggregates of inter-fibrilar mitochondria which were pleomorphic. Many mitochondria showed prominent lesions characterized by swelling, vacuolization, and disintegration of the mitochondrial cristae (Figure 9.2).

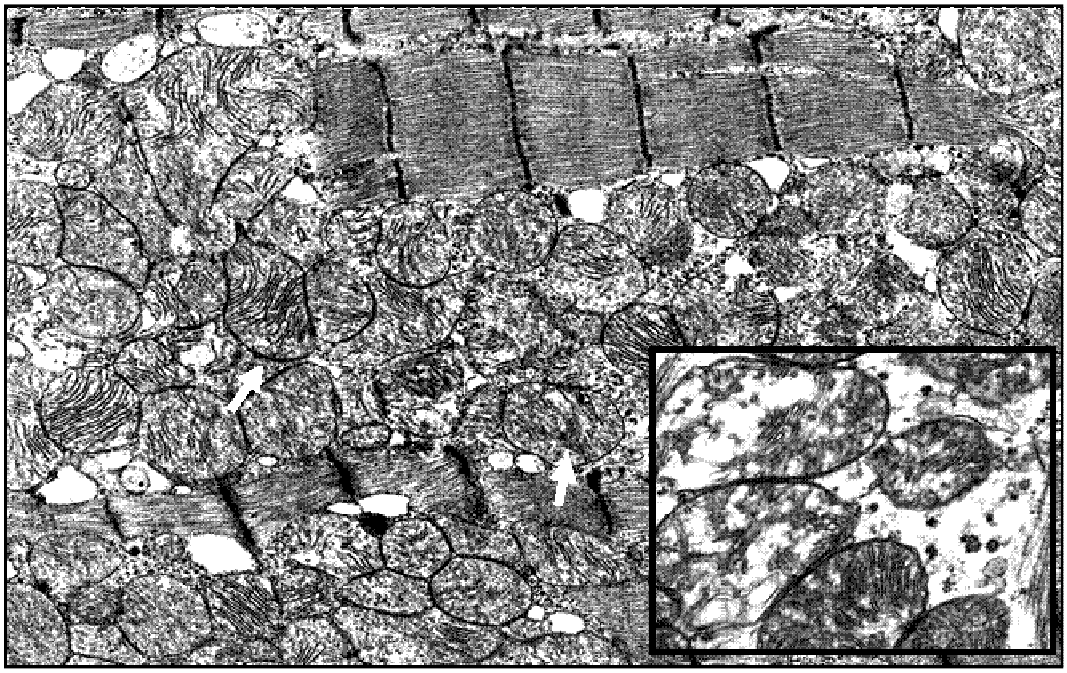

Figure 9.2. Transmission electron microscope image of inter-fibrillar mitochondria in the left ventricular myocardium of a fast-growing broiler chicken. Original magnification 4,500X.
Some cardiomyocytes contained large aggregates of pleomorphic mitochondria (white arrows), with many showing prominent lesions characterized by swelling, vacuolization, and disintegration of the mitochondrial cristae (insert).

The respective levels of cardiac CrP and ATP were lower ($p<0.05$) by 28.8% and 35.7% in birds with fulminant CHF in comparison to slow-growing feed restricted broilers (Table 9.2). ATP and CrP levels in fast-growing *ad libitum* fed broilers were lower as compared to feed restricted broilers, but the differences were not significant ($p>0.05$).

Table 9.2. Comparative study of enzyme (CK, LDH, PDH, and α-KGDH) activities and substrate (CrP, ATP and L-Carnitine) content in the left ventricular myocardium in feed restricted slow-growing broilers (Broiler-Res, low risk of heart failure), *ad libitum* fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with congestive heart failure (Broiler-CHF).

Parameter†	Broiler-Res	Broiler-AL	Broiler-CHF	P Value
ATP	1.641 ± 0.097^{b}	1.353 ± 0.108^{ab}	1.056 ± 0.096^{a}	*p<0.005*
CrP	1.072 ± 0.050^{b}	1.061 ± 0.019^{b}	0.764 ± 0.014^{a}	*p<0.001*
L-Carnitine	0.42 ± 0.093^{b}	0.28 ± 0.09^{ab}	0.17 ± 0.011^{a}	*p<0.04*
CK	12.692 ± 0.565^{a}	16.182 ± 0.022^{b}	15.180 ± 0.284^{b}	*p<0.02*
LDH	2.282 ± 0.126	2.360 ± 0.099	2.550 ± 0.071	*P=0.19*
α-KGDH	0.848 ± 0.053	0.911 ± 0.049	0.941 ± 0.044	*P=0.41*
PDH	0.348 ± 0.015^{b}	0.260 ± 0.013^{a}	0.435 ± 0.007^{c}	*p<0.005*

Values are means ± SE of 5 samples for ATP, CrP, CK, LDH, α-KGDH and PDH, and 4 for L-Carnitine. Means within rows with different superscripts are significantly different ($P< 0.05$). †Units: ATP and CrP (μg/mg of heart tissue); L-Carnitine (μM)/mg heart tissue); CK, and LDH, activities (OD/minute/mg heart tissue); α-KGDH, and PDH activities (OD/minute/g heart tissue)

The CK activity was lower ($p<0.05$) in feed restricted broilers as compared to *ad libitum* fed broilers and birds with fulminant CHF. The LDH activity tended ($p=0.19$) to be higher by 11.8% in birds with CHF as compared to feed restricted broilers. PDH activity was highest ($p<0.05$) in birds with CHF as compared to slow-growing and fast-growing broilers, while no differences in α-KGDH activity ($p>0.05$) were observed. The L-carnitine content in the heart tissue was lower ($p<0.05$) in birds with fulminant CHF as compared to feed restricted broilers. Cardiac L-carnitine tended to be lower in fast-growing broilers but the difference was statistically not significant.

9.5. Discussion

The present study confirmed that rapid growth in broilers is the main precipitating factor of congestive heart failure, as feed restriction at a level 70% of the *ad libitum* fed group eliminated the risk of CHF practically to zero. This is consistent

with the findings of others (Shlosberg *et al.*, 1991; Acar *et al.*, 1995; Olkowski *et al.*, 2007a).

Notably, the slow-growing broilers did not show any sign of heart failure, hence we can argue that the physiological and biochemical parameters in these birds can be used as reference values. Therefore, in order to better understand the mechanisms leading to deterioration of heart function, we compared the values from fast-growing broilers (high incidence and risk of heart failure) and birds with CHF with reference to levels in slow-growing broilers (low risk of heart failure) taken as 100%. These comparisons are presented in Figure 9.3 and 9.4.

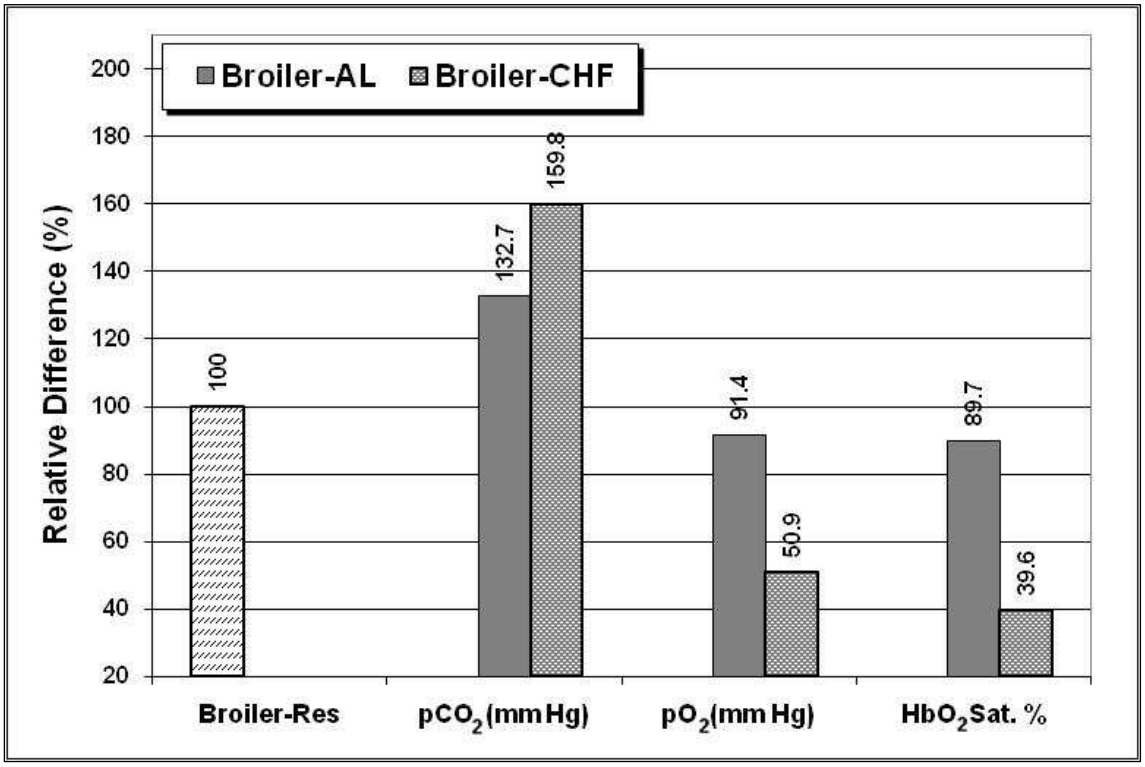

Figure 9.3. Relative levels of blood parameters (pCO_2, pO_2 and HbO_2 Saturation %) in ad-lib fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with congestive heart failure and ascites (Broiler-CHF) expressed on percentage basis of feed restricted slow-growing broilers (Broiler-Res, low risk of heart failure).

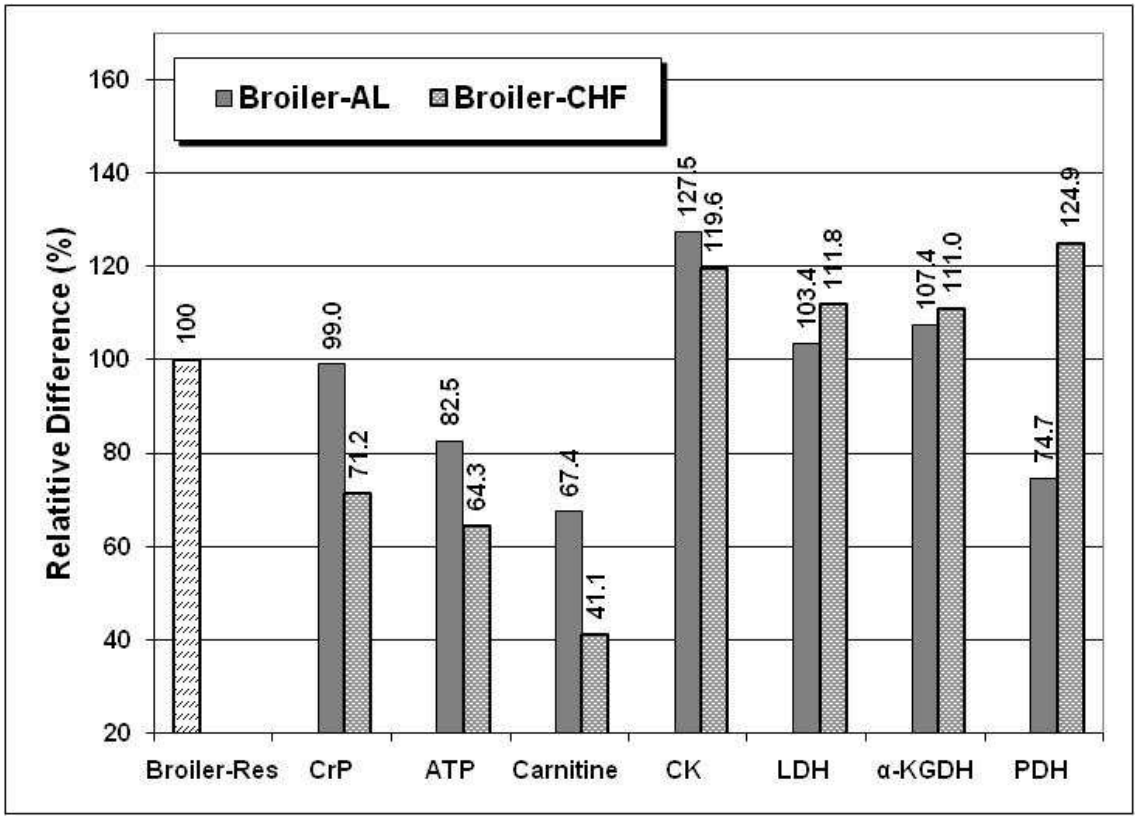

Figure 9.4. Relative levels of substrates (CrP, ATP and L-Carnitine) and enzymes (CK, LDH, PDH, and α-KGDH) activities in ad-lib fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with congestive heart failure (Broiler-CHF, observed within *ad libitum* fed group) expressed on a percentage basis of feed restricted slow-growing broilers (Broiler-Res, low risk of heart failure).

Since all broilers used in the present study were randomly derived from the same population, essentially all categories (i.e. slow-growing, fast-growing, and CHF broilers) represented the same physiological phenotype. Hence, in view of our data, we can argue that the observed systemic physiological changes are associated with metabolic changes in the myocardium. Notably, relative to slow-growing broilers, most of the studied parameters in fast-growing broilers showed trends similar to those seen in broilers with signs of CHF. Therefore it is reasonable to argue that: 1) impairment of energy metabolism in the heart is associated with rapid growth rate in broilers, and 2)

further deterioration of some metabolic function is clearly associated with heart pump failure.

The present study confirmed our previous observation that the deterioration in heart function in fast-growing broilers is associated with lower myocardial energy reserves (Olkowski *et al.*, 2007a). However, the present findings provided new insight into the biochemical mechanisms associated with the decline of cardiac high energy substrates. Interestingly, in comparison to slow-growing broilers, the patterns of activities of some enzymes involved in cardiac energy metabolism were either not changed, or were higher in fast-growing broilers, or those with CHF (Figure 9.4). The observed marked proliferation of mitochondria in affected hearts may indicate a morphological response of the failing myocardium attempting to increase its metabolic potential to provide sufficient energy for heart function. This may help to explain the association of physiological and biochemical changes.

If we consider the findings presented in Figure 9.1, it is clear that impaired heart function (declining HR) is evident in *ad libitum* fed birds, and further deterioration in heart function is associated with CHF. In fast-growing broilers predisposed to heart failure, the heart struggles to meet the oxygen demand requisite for rapid body mass acquisition, and birds become chronically hypoxemic (Olkowski *et al.*, 2005b). As the deterioration of cardiac function progresses to CHF, these signs become more prominent. This was clearly evidenced in our study from blood gas measurements (lowered pO_2, and HbO_2 saturation %) and clinical signs of hypoxemia (cyanosis of combs and wattles). The blood gas profile in this study is consistent with the findings of others (Julian and Mirsalimi, 1992; Buys *et al.*, 1999; Olkowski *et al.*, 1999).

Notably, in both fast-growing broilers and those with CHF respiration rate increased, but the blood gas profiles indicate that the increased respiratory efforts failed to improve blood oxygenation and reduce blood CO_2 level (Figure 9.3). This indicates that hypoxemia in these broilers is not caused by a lack of oxygen, but rather inadequate blood flow in the lungs, which is a direct effect of impaired heart pump function. From

a patho-physiology stand point, only poor myocardial performance which results in poor body tissue and lung perfusion explains the concomitant occurrence of hypoxemia (low pO_2) and hypercapnia (high pCO_2).

The loss of contractile function of the heart is associated with mitochondrial inability to supply ATP to the myocardium, leading to a state of energy deprivation in the heart (for review see Stanley *et al.,* 2005). Findings from our study correspond with those from our earlier study in which we reported that deterioration of heart function is associated with overall low cardiac energy reserves such as ATP and CrP (Olkowski *et al.,* 2007a). Similar observations have been made in human and animal models of cardiomyopathy (Liao *et al.,* 1996; Hansch *et al.,* 2005; Nakae *et al.,* 2005). Reduced ATP synthesis has been demonstrated in isolated cardiac mitochondria from failing hearts (Sharov *et al.,* 1998; 2000). The metabolic mechanisms responsible for this lowered energy pool involve both energy synthesis and transformation pathways.

Chronic hypoxemia leads to tissue hypoxia, and in an anaerobic situation, the glycolytic pathway supplies substrate for LDH and contributes to energy synthesis. Careful analysis of data presented in Figure 9.4 revealed a noticeable trend in the LDH being lowest in slow-growing broilers (low risk of heart failure), increasing in *ad libitum* fed birds (high risk of heart failure), and being highest in birds with CHF. Although the trend was not statistically significant, it does suggest that LDH activity is associated with increased susceptibility to heart failure. Our observations are consistent with findings reported in other studies. Biopsy samples taken from patients suffering from congestive heart failure also revealed higher activity of LDH (York *et al.,* 1976; Schultheiss *et al.,* 1980).

The activity α-KGDH was higher in hearts from fast-growing broilers and those with CHF, which suggests that this pathway does not account for the lower ATP content in fast-growing broilers, and is not a direct cause of heart failure in broilers with CHF. Increased activity of α-KGDH and PDH [the rate limiting enzymes of the tri-carboxylic acid (TCA) cycle], in the birds with CHF suggests that this pathway is not likely the

direct cause associated with the lower energy pool observed in fast-growing broilers and in birds with CHF. Rather, the enhanced activity levels of these enzymes suggest that affected birds are trying to compensate by providing additional metabolic resources to metabolize pyruvate generated from glycolysis. Proliferation of myocardial mitochondria may represent morphological adaptation of the heart to biochemical changes in cardiomyocytes.

Enhanced proliferation of mitochondria and elevated TCA enzyme activities may represent a compensatory response to the observed lower myocardial L-carnitine levels in both fast-growing broilers (high risk of heart failure) and those with CHF. L-carnitine, a water soluble quaternary amine, is of critical importance in cardiac energy supply metabolism by virtue of controlling the influx of long chain fatty acids into mitochondria via the carnitine acyltransferase enzyme system (Arenas *et al.,* 1998). It also traps potentially toxic acyl-CoA metabolites, which may increase during acute metabolic crises through esterification reactions that may impair the TCA cycle and fatty acid oxidation pathways (Tein, 2003).

Therefore, in the context of cardiac energy metabolism, the observed lowered myocardial L-carnitine in fast-growing broilers and birds with CHF is of metabolic significance. The reduced L-carnitine levels in broilers would inevitably lead to a reduction in fatty acid oxidation. Given that β-oxidation of fatty acids contributes 60 to 90% of energy substrate production in cardiomyocytes (Stanley *et al.,* 2005; Neubauer, 2007), inadequate levels of cardiac carnitine may explain low cardiac energy status in these birds. L-carnitine deficiency is associated with cardiomyopathies in human patients (Hirata *et al.,* 1986; Colin *et al.,* 1987). Furthermore, L-carnitine supplementation reduced incidence of CHF in broilers (Geng *et al.,* 2004).

An important component of cardiac energy management is the transfer of energy in the myocardium by the creatine kinase system. In a normal physiological situation, CK catalyzes the transfer of the high-energy phosphate bond from ATP to creatine to form CrP and ADP and vice versa. CrP can pass rapidly through the mitochondrial

membranes and act as an instant source of ATP in the myocardium. Interestingly, slow-growing feed restricted birds showed lower CK activity than fast-growing birds. This suggests that CK and CrP are essential as a energy reserve in fast-growing broilers as these birds are attempting to compensate for the lower ATP levels by increasing CK activity. Inadequacy of this pathway may be an important factor in the progression of heart failure. CHF may occur in birds that are unable to increase CK activity to a level required to support cardiac function in fast-growing birds (Figure 9.4). Decreased CK activity was found in cardiac tissue of individuals with CHF (Bessman and Carpenter, 1985; Khuchua *et al.,* 1992; Nascimben *et al.,* 1996). Hence, lowered CK activity in birds with fulminant CHF, which already have lower ATP and CrP levels (Olkowski *et al.,* 2007a), may represent a significant metabolic weakness predisposing broilers to heart failure.

The findings from the present study provide further insights into the possible biochemical mechanisms associated with the predisposition of chickens selected for rapid growth to heart failure. Insufficiencies of ATP and CrP appear to be the major factors directly responsible for deterioration of heart function in fast-growing *ad libitum* fed broilers. Regeneration of ATP from the CK pathway appears to be an important factor in cardiac energy management in fast-growing but not in slow-growing broilers. Lower myocardial L-carnitine levels of *ad libitum* fed broilers and birds with fulminant CHF provide evidence that low energy substrate levels may be associated with either insufficient transport of fatty acids for the β-oxidation pathway or shift in metabolism to favour carbohydrate metabolism instead of fatty acid metabolism. Further investigations into the role of fatty acid oxidation capacity and other potential causes for myocardial energy imbalance, such as electron transport chain function, are warranted.

10. VASCULAR REMODELING AND ITS ROLE IN THE PATHOGENESIS OF ASCITES IN FAST-GROWING COMMERCIAL BROILERS

10.1. Abstract

Ascites is a condition commonly observed in fast-growing commercial broilers, resulting in significant economic losses to broilers industry. Previous observations from our lab revealed that in addition to heart pathology, ascitic broilers show gross signs of blood vessel pathology. This study examined heart pathology in the context of the putative role of blood vessel pathology associated with development of ascites in broilers. Samples for microscopic and ultra structural study were obtained from commercial male broiler chickens randomly selected from a basic flock representing feed-restricted slow-growing broilers, fast-growing *ad libitum* fed broilers, and broilers that developed ascites within *ad libitum* fed group. Arteries were subjected to histo-pathological examination of elastic components using a special stain (HOPS). Scanning electron micrographs were obtained from posterior vena cava to evaluate extracellular matrix and fibrilar collagen remodeling in these three groups of broilers.

Grossly, the arterial walls from ascitic broilers appeared flaccid and lacked elasticity. Histo-pathological evaluation of major arteries from ascitic broilers showed loss and thinning of elastic elements. The findings from vena cava in broilers with CHF and ascites revealed reduced network density of the structural matrix as well as increased thickness of fibers. From our findings, it can be inferred that the structural changes seen in the arteries from ascitic broilers are maladaptive, such changes would definitively increase hemodynamic burden, and thus have a detrimental effect on the heart pump performance. The changes in veins from ascitic broilers are indicative of pathological remodeling conducive to increased permeability of the vascular wall. Taken together, hemodynamic burden associated with heart pump failure would provide

conditions conducive for seepage and accumulation of ascitic fluid. Our findings indicate that blood vessel pathology plays an important role in the pathogenesis of ascites in broilers.

10.2. Introduction

Ascites is abnormal accumulation of fluid in abdominal cavity, commonly observed in commercial broilers. Globally, the economic losses to the broiler industry from ascites have been estimated to be around one billion dollars annually (Maxwell and Robertson, 1997). Despite considerable research efforts, the pathogenesis of ascites in fast-growing broilers is poorly understood.

One of the most pathognomonic change observed in broilers with congestive heart failure (CHF) and ascites is dilation of left and right ventricular chambers (Nain *et al.,* 2008b; Olkowski *et al.,* 2007a). Previous studies conducted in our lab showed that many fast-growing broilers show dilated heart pathology and clinical signs of CHF, but not all of these broilers develop ascites (Olkowski *et al.*, 1998; 1999; 2003b). Interestingly, in some instances broilers with moderate dilation of left and right ventricles developed massive ascites, whereas many broilers with similar or even more severe heart pathology and signs of CHF do not develop ascites. Hence, these observations raised the possibility that, in addition to heart pathology, there are other essential factors determining whether the outcome is CHF with or without ascites.

Closer evaluation of our data revealed some noteworthy trends. We noted that ascitic broilers, in addition to gross heart pathology, in most cases, consistently show changes that are indicative of pathological remodeling of the major blood vessels (Olkowski and Wojnarowicz, unpublished observations). Clearly, in the shadow of the tenets centered on pathology of the myocardium, vascular changes have not received adequate attention. We hypothesized that vascular changes may play an important role in the development of ascites in broilers.

Accordingly, our interest was primarily focused on detailed examination of the morphological features in the major blood vessels. Since ascites in broilers is clearly associated with rapid growth, whereas when the growth rate is controlled, the risk of ascites decreases dramatically (Acar *et al.*, 1995; Olkowski and Classen, 1998b; Nain *et al.*, 2008b), we were interested in comparing the blood vessel morphology in fast-growing broilers (high risk of ascites), slow growing broilers (low risk of ascites) and broilers with ascites.

10.3. Materials and Methods

10.3.1. General

In a retrospective context, the information on gross pathological changes in the major blood vessels examined in this study were compiled based on the extensive post mortem data base collected in our lab over the last several years. In a prospective study, in order to further investigate the vascular remodeling associated with ascites, arterial vessels (aorta, brachiocephalic and pulmonary arteries) and posterior vena cava were obtained from slow-growing feed-restricted broilers fed with diet equal to 70% of the *ad libitum* fed broilers (low risk of congestive heart failure (CHF) and ascites), fast-growing broilers fed *ad libitum* (high risk of CHF and ascites), and from broilers that developed ascites. The details of experimental designs, management, feeding regime, and environmental conditions were described previously (Olkowski *et al.*, 1999; 2003; 2005b; Nain *et al.*, 2008b). Experimental protocols were approved by the University of Saskatchewan Animal Care Committee and procedures were performed in accordance with the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

10.3.2. Clinical evaluation

The birds were monitored daily for presence of overt signs of impending CHF such as exercise intolerance, tachypnea, cyanosis of combs and wattles, distended abdomen, and presence of ascitic fluid. Those broilers with the above mentioned signs were euthanized and subjected to detailed post mortem examination.

10.3.3. Post Mortem Examination

Detailed gross post-mortem examination was performed on all mortalities, euthanized birds and all remaining broilers upon termination of the study. The diagnosis of CHF and ascites was based on gross dilation of the ventricular chambers along with abnormal accumulation of ascitic fluid in the abdominal cavity.

10.3.4. Light Microscopy

The aorta, brachiocephalic and pulmonary arteries were obtained during 6^{th} week of age from three randomly selected apparently normal *ad libitum* fed broilers, and three ascitic broilers observed within *ad libitum* fed group, and processed for microscopic examination immediately after cervical dislocation. The specimens were fixed in 10% buffered formaline, and following fixation embedded in paraffin. Transverse sections (5 μm) from the arteries were processed for light microscopy and stained with haematoxylin/orcein/phyloxin/saffron (HOPS), which is considered as specific stain for elastic elements in blood vessels.

10.3.5. Scanning Electron Microscopy

Samples of posterior vena cava were fixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer, and were further processed as described previously by Olkowski *et al.* (2001). Briefly, fixed samples were washed with de-ionized water. These samples were subsequently dehydrated in graded concentration of acetone and subsequently washed with 100% acetone thrice, each for a 5 minute duration. The dehydrated samples were freeze dried and subsequently mounted on aluminum stubs with exposed longitudinal fractured surface and finally spatter coated with gold. The gold coated samples were examined under scanning electron microscope JEOL 840A.

10.4. Results

Based on retrospective data, dilated cardiomyopathy is observed in many fast-growing broilers upon post mortem examination, but approximately 15 to 25% of broilers with clinically significant heart pathology do not develop ascites. Also, our long term observation showed that controlling growth rate with feed restriction practically eliminates heart pathology in broilers.

In the prospective study, no cases of CHF, with or without ascites, were observed in the feed restricted group, while in the *ad-libitum* fed groups approximately 60% of broiler revealed gross dilation of ventricular chambers upon post mortem examination. Among these cases, approximately 77% showed signs of ascites.

All broilers showing signs of CHF and ascites revealed abnormal accumulation of ascitic fluids in the abdominal cavity along with gross dilation of the ventricular chambers, nodular changes on the edges of the atrioventricular valve and pericardial effusion. Many broilers showing similar or even more severe signs of CHF and heart pathology did not develop ascites. In addition to gross changes in the heart, ascitic broilers showed signs of vascular remodeling in major vessels. Such vascular changes were not apparent in broilers showing signs of CHF without ascites, in normal fast-growing *ad libitum* fed broilers, or in slow-growing feed restricted broilers.

On cross section, grossly, the arteries from normal broilers and those showing dilated cardiomyopathy without ascites appeared circular, with firm vascular wall tone, characteristics of the normal artery (Figure 10.1a). In contrast, the arterial walls from ascitic broilers appeared thin, flaccid and lacked elasticity, which was clearly evidenced by ellipsoid shape of cross-sectional arterial lumen, or in many cases collapsing of vessel, owing to the structural weakness of the arterial walls (Figure 10.1b).

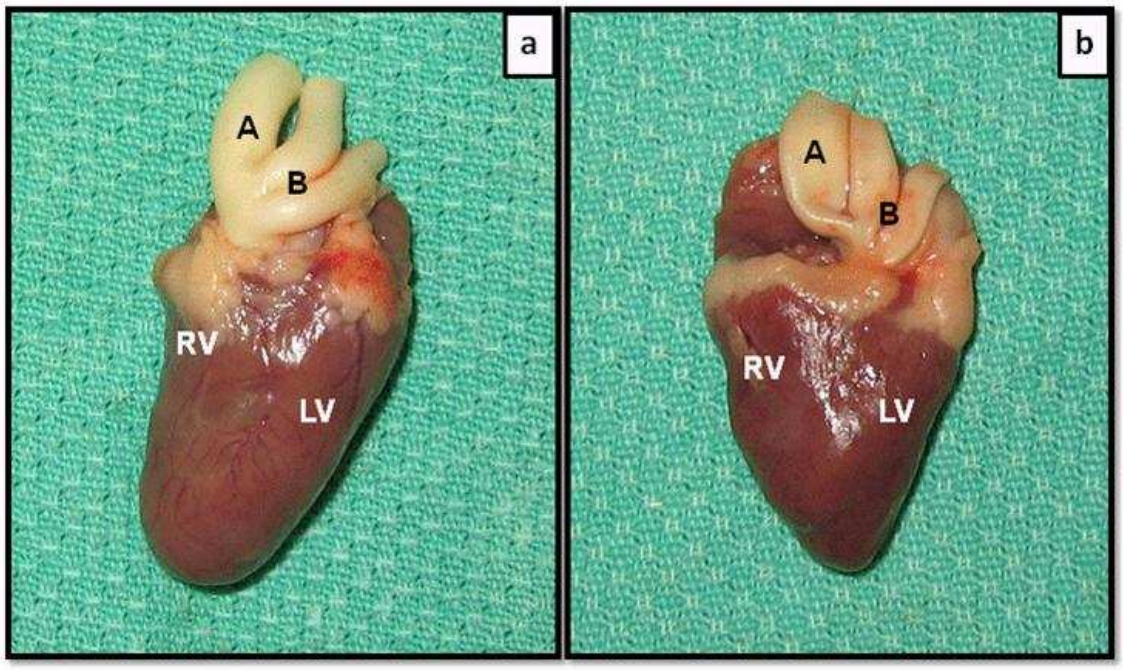

Figure 10.1. Blood vessels morphology in an apparently normal fast-growing ad libitum fed broiler (a) and in a broiler with ascites (b).
Cardiac structures are identified as follows: LV (left ventricle), RV (right ventricle), A (aorta), B (brachiocephalic arteries). Comparatively to the apparently normal broiler, the hearts form the ascitic broiler show evidence of moderate ventricular dilation. Noteworthy are considerable differences in tone of major vessels. Aorta and brachiocephalic arteries in normal broiler appears firm and show normal tone characteristic. In contrast, the vessels from the ascitic broiler appear thin and flaccid, and are collapsed.

Histo-pathological evaluation of these arteries revealed that vessels from ascitic broilers showed substantial changes in vascular wall architecture characterized by thinning, or occasionally total loss of elastic elements (Figure 10.2 and 10.3). Qualitatively the changes were similar in aorta, brachiocephalic arteries, and pulmonary arteries. In normal broilers, the intima was tightly packed with a mat of elastic fibers, whereas in the ascitic broiler, the elastic fibers appeared sparse and separated by numerous, vast clear spaces. The tunica media of the healthy broiler consisted of numerous dark staining and heavily interwoven smooth muscles, whereas the smooth muscles of the ascitic broiler showed wispy pale pink cytoplasm.

The most drastic difference in arterial wall architecture between normal and ascitic broilers was in the number and visual qualities of the elastic fibers of the tunica media. In the healthy broiler, the elastic fibers are numerous, thick and heavily interwoven. They readily form continuous mats of fibers on the surfaces of the smooth muscles. The elastic fibers in the ascitic broiler are scant and thin. As a result, the cellular walls of the smooth muscles often appear free from adhering elastic fiber mats. The tunica adventitia in a healthy broiler continues to display a rich tangle of elastic fibers while the elastic fibers in the vessels of an ascitic broiler are rarefied and predominantly linear.

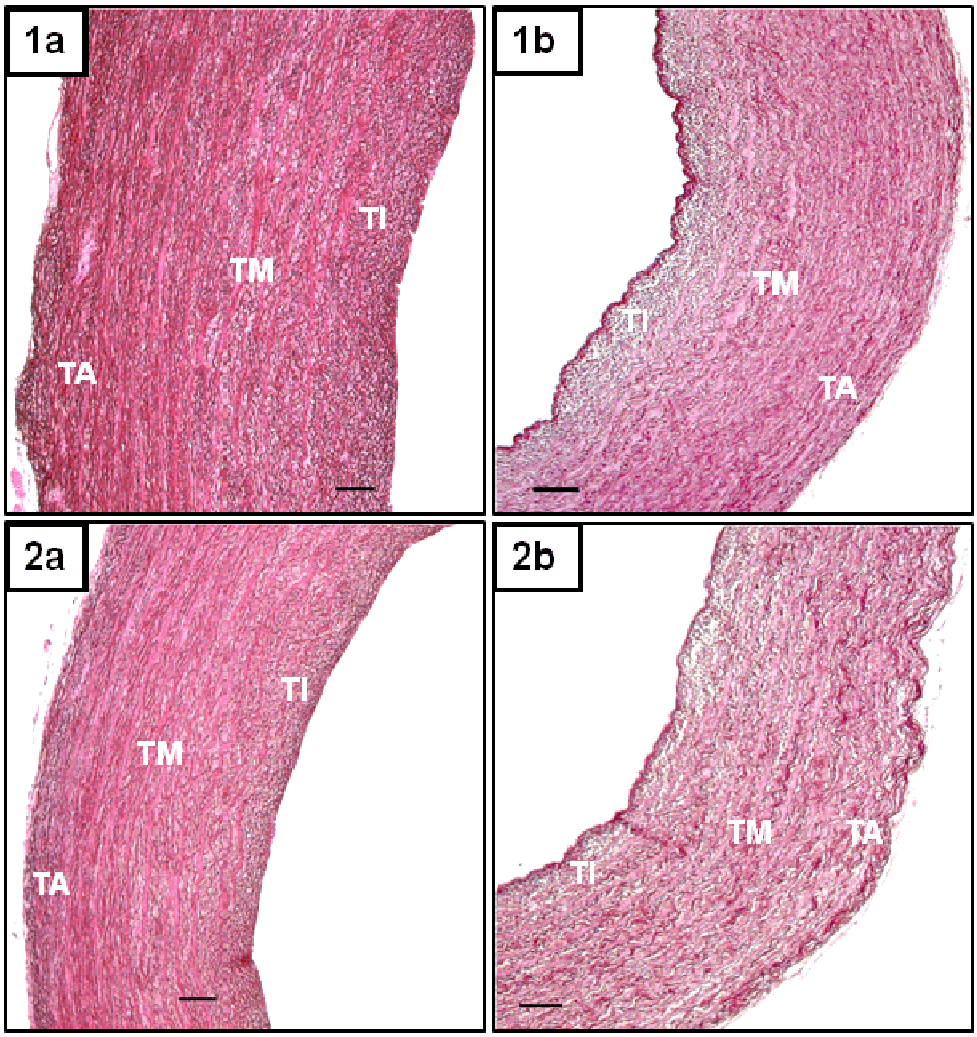

Figure 10.2. Cross section of aorta (1a) and brachicephalic arteries (2a) from an apparently normal fast-growing ad libitum fed broiler and from a broiler with ascites (1b and 2b, respectively).
Vessel wall structures are identified as follows: TI (tunica intima), TM (tunica media), TA (tunica adventitia). Notably, in apparently normal *ad libitum* broilers both aorta and brachiocephalic arteries (Figs. 1a and 2a respectively) the intima appears tightly packed with a mat of elastic fibers. In contrast, these vessels in the ascitic broiler (1b and 2b) show intima with sparsely distributed elastic fibers punctuated by numerous clear spaces. The tunica media of the healthy broiler consists of numerous dark staining and heavily interwoven smooth muscle, whereas the smooth muscles of the ascitic broiler show wispy pale pink cytoplasm. The tunica adventitia in a healthy broiler shows a rich tangle of elastic fibers, while this structure in the vessels of an ascitic broiler appears rarefied and predominantly linear. Tissue was stained with haematoxylin/orcein/phyloxin/saffron (HOPS), which is considered as specific stain for elastic elements in blood vessels. Bar = 150 µm.

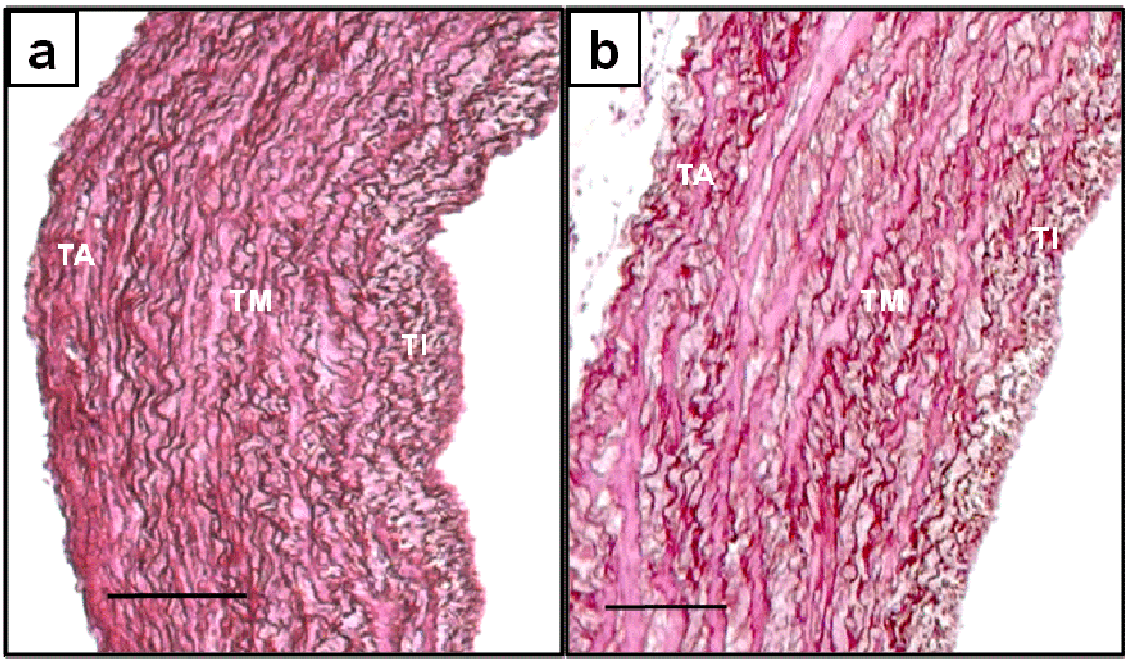

Figure 10.3. Cross section of pulmonary arteries from an apparently normal fast-growing ad libitum fed broiler (a) and from a broiler with ascites (b).
Vessel wall structures are identified as follows: TI (tunica intima), TM (tunica media), TA (tunica adventitia). Notably, in the apparently normal fast-growing *ad libitum* fed broiler pulmonary arteries (a) the intima appears tightly packed with a mat of elastic fibers, whereas in the ascitic broilers (b) intima shows sparsely distributed elastic fibers punctuated by vast clear spaces. The tunica media of the healthy broiler consists of numerous dark staining and heavily interwoven smooth muscle, whereas, the smooth muscles of the ascitic broiler show wispy pale pink cytoplasm. The tunica adventitia in an apparently broiler shows a rich tangle of elastic fibers, while this structure in ascitic broiler appears rarefied with numerous clear spaces. Tissue was stained with haematoxylin/orcein/phyloxin/saffron (HOPS). Bar = 150 µm.

The findings from vena cava in broilers with ascites revealed a considerably reduced network density of the structural matrix of the vascular wall, as well as increased thickness of fibers. The representative images from scanning electron microscope of vena cava are demonstrated in Figure 10.4. Noteworthy are the striking differences in vascular wall architecture between normal broilers and broiler with ascites, with a considerably reduced network of extra-cellular fibers, as well as increased thickness of fibers. It is also of interest to note that, if we compare venous wall architecture between slow-growing feed restricted broilers (low risk of ascites),

fast-growing *ad libitum* fed broilers (high risk of ascites), and broilers that developed ascites, there is a clear pattern where decreasing density of thin weave of collagenous fiber appears to be correlated with the risk of ascites.

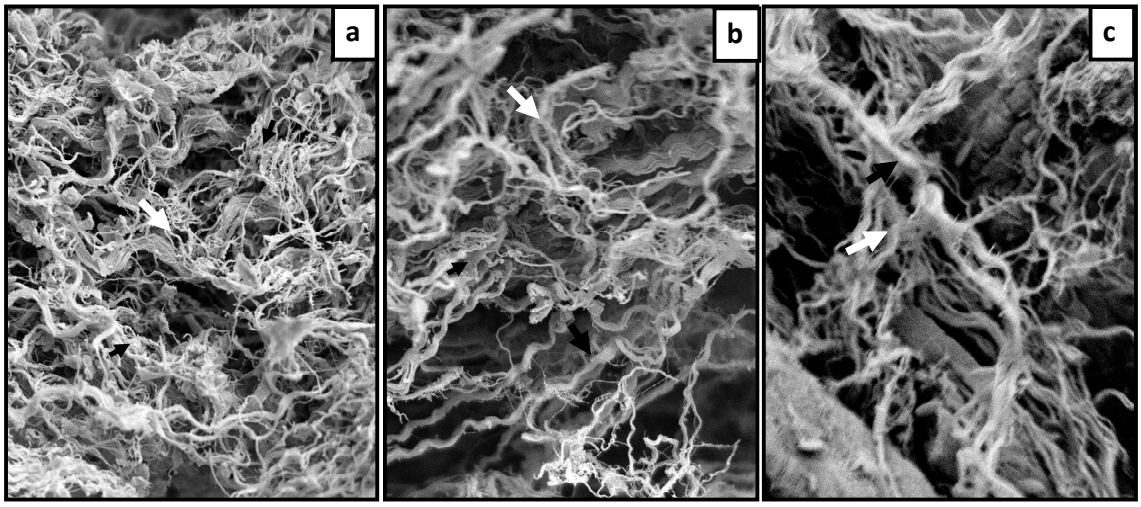

Figure 10.4. Scanning electron micrograph of the vessel wall from posterior vena cava of a feed restricted slow growing broiler (a), a fast-growing ad-lib fed broiler (b) and a broiler with ascites (c).
Noteworthy are differences in vessel wall architecture between slow-growing, feed restricted broiler (a), apparently normal fast-growing, *ad libitum* fed broiler (b), and ascitic broiler (c). In slow-growing broiler (low risk of ascites), the elastic matrix (Figure 10.4a, arrow) consist of well-defined, fine and dense weave of elastic struts. In contrast, in fast-growing broiler (high risk of ascites) the mesh of elastic elements in the vessel wall is considerably reduced, and individual struts appear thicker (Figure 10.4b, arrow) in comparison to slow growing broiler. In the ascitic broiler, the fine network of elastic elements struts appears disrupted, and the pattern of dense and fine weave is replaced by thick and sparsely populated elements (Figure 10.4c, arrow).

10.5. Discussion

By definition, ascites is the accumulation of fluid in body coelomic cavities, and as such it is a non specific sign that may be associated with several causes. In broiler chickens, common ascites cases are most often associated with heart failure. The hemodynamic burden in the venous part of the circulation that contributes to the development of ascites in broilers may be associated with several patho-physiological variables of cardiogenic origin. Those that have already been documented include: 1)

atrio-ventricular valve insufficiency (Olkowski *et al.*, 1998; 2005a), 2) poor performance of the heart pump evidenced by declining heart rate and decreased fractional shortening (Olkowski and Classen, 1998; Deng *et al.*, 2006; Druyan *et al.*, 2007). The present study revealed that, in addition to cardiogenic causes, the hemodynamic changes associated with pathological remodeling of the major blood vessels ought to be considered in the pathogenesis of ascites.

Elastic arteries distribute blood to the whole body and act as a cushion for pulsatile blood flow (Milnor, 1984). This cushioning effect provided by these arteries results in reduced ventricular after-load and steady blood flow. It has been observed that changes in the left ventricular after-load affects the cardiac performance and have serious hemodynamic effects (Laskey *et al.*, 1985).

The loss of functional elasticity affects the wall stress and ultimately cardiac after-load. It has been demonstrated that impaired synthesis of elastin in the wall of the aorta leads to altered mechanical properties and subsequently increased left ventricular mass and cardiovascular diseases (Martyn and Greenwald, 1997). Such changes in properties of the aorta may have a negative influence on the pulsatile function of the failing heart (Pepine *et al.*, 1978). It is interesting that findings from patients with CHF revealed decreased cardiac output and increased systemic vascular resistance (Laskey *et al.*, 1985).

Findings from the broilers with ascites indicate that major vessels in these broilers undergo pathological remodeling. The observed changes suggest decreased elasticity and lower tensile strength of the affected vessels. Blood vessels play an important role in blood circulation. Undoubtedly, the loss of vessel tone and elasticity in the main arteries observed in ascitic broilers would have a major impact on heart function. Elastic recoil of major arteries transfers the kinetic energy generated during ventricular systole, and pulsatile propagation of this energy is essential for sustained circulation of blood throughout the body. Elasticity of vessels is also an important determinant of the blood pressure within a vessel. The structural changes in the conduit

arteries and veins can affect the ventricular preload and after-load and ultimately will have a detrimental effect on the heart pump efficiency.

Patients with congestive heart failure display elevated pulsatile load (Mitchell *et al.*, 2001). The input impedance of the arterial system consists of systemic vascular resistance, total arterial compliance and wave reflections (Nichols and O'Rourke, 1990). The input impedance of the arterial system has been described as a major determinant of stroke volume from the left ventricle (Maughan *et al.*, 1984). Recently, Curtis *et al.* (2007) demonstrated that energy of the forward compression wave generated by the left ventricular wave (responsible for blood flow through arteries) decreases, but reflection of this wave increases in patients with congestive heart failure. Increased wave reflection adds an additional load on cardiac function during heart failure.

Poor performance of the heart is inherently associated with rapid growth in broilers (Olkowski and Classen, 1998b; Korte *et al.,* 1999; Deng *et al.,* 2006; Druyan *et al.,* 2007), thus loss of arterial wall elasticity may further compromise heart pump performance of an already failing myocardium, and ultimately hasten the development of CHF. However, in this context the factors determining whether the outcome of heart pump failure is CHF with or without ascites require further deliberation.

Notably, the present study, in agreement with our previous observation (Olkowski *et al.,* 1998; 1999; 2003b) showed that many fast-growing broilers showing clinical signs of CHF and severe heart pathology on post mortem examination do not develop ascites. This indicates that neither severity of clinical signs nor pathological changes in the heart, are clearly predictive of ascites. However, because the vast majority of broilers with ascites, in addition to heart pathology, show changes in major vessels, it is reasonable to speculate that pathological remodeling of vessels is an essential factor involved in the pathogenesis of ascites.

Ascites is the accumulation of fluid in body coelomic cavities and vessel permeability may be a key factor arbitrating the migration of plasma from the vascular

bed to body spaces. Fluid seepage across the vessel wall is determined by three major variables: 1) hydrostatic blood pressure inside the vessel which tries to force the fluid out, 2) integrity and tension in the vessel wall resisting the outflow, and 3) colloidal osmotic pressure. The inevitable result of a failing heart pump is a hemodynamic state where the blood flow is sluggish or in more advanced stages, even transient stagnation of blood in the venous side of the circulation may occur. Ultrastructural changes in vena cava from ascitic broilers can be characterized as pathological remodeling with loss of thin and dense weave of elastic collagenous fibers, which are replaced by a considerably less dense and thicker weave of fibers. Such architectural changes in the venous wall would have major effects on the tensile strength of the vessel as well as on the structural density of the vessel wall. In this context, it is noteworthy that there are remarkable differences in the venous wall matrix density between slow growing broilers (low risk of ascites) and fast-growing broilers (high risk of ascites). Hence it is possible that pathological remodeling observed in the veins of fast-growing broilers, when coupled with the developing heart pump failure, may be an important factor facilitating development of ascites.

The findings from the present study indicate that structural changes observed in the arteries of ascitic broilers are maladaptive and can lead to increased left and right ventricular preload and may have a detrimental effect on heart performance, while the changes in veins are indicative of pathological remodeling conducive to increased permeability across the venous walls, and thus accumulation of ascites. The present study provides evidence that vascular pathology is an integral element in the etiology of ascites in fast-growing broilers. Impact of the observed changes on blood flow dynamics should not ignored. In order to fully understand the mechanisms leading to development of ascites in broilers, the bio-mechanical effects of pathological remodeling in the wall of blood vessels on heart function, blood flow dynamics, and vascular wall integrity, as well the causes of vascular pathology need to be further investigated.

11. GENERAL DISCUSSION AND CONCLUSIONS

Heart related problems are a major concern in the broiler industry leading to significant economic losses. Modern fast-growing broilers are inherently predisposed to increased risk of heart failure. This increased predisposition is subsequent to broilers altered physiology, a result of genetic selection. The presence of any cardiotoxic compounds under this increased risk can precipitate heart conditions in susceptible broilers. The dietary factors that have the ability to precipitate or increase the risk of heart failure in broilers include over-supplementation of vitamin A and D_3, and methanol soluble factors present in meat meal (MM). On the other hand, vitamin E (antioxidant vitamin) has no effects on incidence of heart failure in broilers, while vitamin C appears to delay the onset of heart failure in broilers.

Acute heart failure develops rapidly and is considered to be a life threatening event as the heart function stops abruptly. Ventricular arrhythmia is the critical step in the development of acute heart failure that may degenerate to ventricular fibrillations. In the present study, only vitamin D_3 was found to be a risk factor for acute heart failure in broilers. Stimulated stress findings revealed that ventricular arrhythmia was of greater magnitude and duration in broilers fed vitamin D_3 fortified diet. This increased risk of arrhythmia was most probably due to the presence of degenerative changes in the myocardium and more specifically in His-Purkinje system. These changes in myocardial tissue may act as a contributing factor to arrhythmogenesis that may trigger fatal cardiac arrhythmia in broilers already at higher risk of electro-physiological instability associated with fast growth. Commercial broilers are subjected to various forms of environmental stress e.g. overcrowding, antagonistic behavior, procedural and/or routine management activities etc. These stressful events will certainly evoke adrenergic response and this adrenergic response is most likely to be similar to the stimulated stress challenge used in the present study. Hence, it is possible that excess of

vitamin D_3 may further sensitize the broiler's myocardium to the effect of stress, under these conditions even mild arrhythmic episodes may deteriorate to life threatening arrhythmia.

Congestive heart failure is a complex syndrome of several dysfunctional mechanisms and is a contuning process rather than a single event resulting in a progressive decline in ventricular contractile function. During this progressive decline in heart function, any insult on the cardiovascular system by any extrinsic factor can precipitate these already existing conditions. Here, under the model used in present study i.e. male broilers under lowered brooding temperature protocol, entire population was at increased risk of heart failure, several factors were identidied that can activate this event in broilers.

The findings from the present study revealed that excess of vitamin A or D_3 acts as an independent risk factor for CHF, and can precipitate heart conditions in susceptible broilers and increase the risk of CHF. Histological examination of the myocardium from apparently normal broilers irrespective of dietary treatment revealed various degenerative changes in the myocardium but the magnitude of these lesions were more apparent in broilers fed with vitamin A or D_3. Most of the studies on vitamin A overexposure are limited to embryonic cardiac malformations (Osmond *et al.,* 1991; Kraft and Juchau, 1993; Mulder *et al.,* 2000; Millemann *et al.,* 2007). The findings from the present study provide a direct link between over-exposure during post embryonic life and risk of CHF in susceptible broilers. These findings provide proof that over-supplementation of vitamin A or D_3 in broilers diet may increase the risk and incidence of CHF. As supplementation of these vitamins is commonly practiced in broilers diet for variety of reasons, if over-supplemented, they are able to precipitate heart conditions in susceptible individuals. Further studies to establish safe levels of dietary vitamin A or D_3 are warranted.

During the course of this study on several occasions it was demonstrated that oxidative stress is generated and involved in the pathogenesis of congestive heart failure

in broilers. The morphological changes observed in cardiac mitochondria (mitochondrial vacuolization and disintegration) in *ad libitum* fed broilers and in broilers with CHF may be subsequent to lipid peroxidation of mitochondrial membranes. Furthermore, *in vitro* studies provide the proof that the generated oxidative stress has the ability to alter the activity of some of the key enzymes involved in the energy synthesis (α-KGDH) and transformation pathways (CK), and may be the reason for insufficiency of ATP and CrP in the myocardium. Additionally, activation of LDH enzyme by ROS may counteract the negative effect of above mentioned enzymes on energy synthesis pathways.

The vitamin E and C, due to their antioxidant property, are believed to have preventive role in the development of CHF. Interestingly, in the present study, dietary supplementation of vitamin E had no beneficial effect on the incidence of heart failure in broilers, although it appears to prevent oxidative damage in normal broilers. However, vitamin C appears to have some beneficial effect in preventing congestive heart failure as revealed by clinical, mortality/morbidity and histo-pathological findings. The observed effect of vitamin C may be due to its hydrophilic nature and wider distribution in the cellular compartments or due to its ability to work as cofactor in the synthesis of catecholamine, collagen and L-carnitine synthesis, is not known at this point of time and needs to be investigated further.

Findings from the meat meal extract study provides the evidence that MM used in commercial broilers diet contains compounds capable of inducing profound morphological and biochemical changes in myocardium and increases the risk of heart failure. The extraction procedure used in the present study was mainly targeted for heterocyclic amines, however the possibility of other methanol soluble factors can not be ruled out. Additional studies are needed to identify individual cardiotoxic compounds, establish safe maximum levels of inclusion, and to develop rendering processing guidelines and quality control.

During the course of this study, another interesting observation was that vessels coming out of the heart were lacking tone and collapsing in broilers developing ascites. Moreover, in some instances it was observed that broilers with moderate dilation of ventricular chambers develop ascites, whereas some broilers with even severe dilation of ventricular chambers do not develop ascites. The broilers developing ascites, in addition to gross ventricular chamber enlargement, consistently showed changes indicative of pathological remodeling of the major blood vessels. These findings suggest that vascular changes had not received adequate attention in the shadow of the pathological changes of the myocardium. Additionally, venous vessels with observed pathological changes would most likely to be susceptible to increased permeability under the condition of volume overload subsequent to congestive heart failure. Hence, hemodynamic burden associated with CHF provides ideal conditions for seepage and accumulation of ascitic fluid. These findings suggest that vascular remodeling takes place during the pathogenesis of ascites in broilers. Further studies are needed to identify the molecular mechanisms associated with vascular remodeling.

Investigators have developed numerous animal models to study congestive heart failure. The most common of them is cardiac pacing canine model. There are few disadvantages of this canine model i.e. it is expensive to develop, time investment and number of animals are limited. There is no such disadvantage with this chicken model of heart failure. Since, there is a marked homology between mammalian and avian species at molecular, biochemical and ultrastructural mechanism of heart failure. Hence, this model offers tremendous potential to be used to test different dietary ingredients for there potential beneficial or adverse effects on cardiovascular system.

Findings from the present study revealed that the outcome of CHF was the same irrespective of the dietary factors i.e. lowered energy reserve and higher lipid peroxidation in the myocardium. Given the fact that broilers are highly predisposed to heart conditions, the over-supplementation of vitamin A or D_3 and methanol soluble factors present in meat meal can result in higher incidence of heart failure. Hence, broilers are inherently predisposed to heart conditions and any slight insult by above

mentioned dietary factors can exacerbate the pre-existing conditions and increase the risk of heart failure in broilers.

A number of questions remain unresolved and needs to be investigated further as highlighted above with the major findings. Most importantly, the lowered CrP and ATP were observed in broilers developing CHF irrespective of the dietary treatments. This insufficiency of energy substrates was not explained by the observed higher activity of selected cytosolic (LDH) and mitochondrial (PDH, KGDH) enzymes. Further studies are needed to determine the exact cause of lowered energy status by looking at the electron transport chain components and enzymes involved in β-oxidation of fatty acids.

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